THE TRANSPORT OF METHYLGLYOXAL BIS(GUANYLHYDRAZONE) AND ITS ROLE IN THE DEVELOPMENT OF RESISTANCE IN MURINE LEUKEMIA L1210 CELLS

BY

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This dissertation is dedicated to my parents, Mr. Tongsuk and Mrs. Tongkam Kanthawatana.

TABLE OF CONTENTS

	page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	ix
CHAPTERS	
1 INTRODUCTION	1
Specific Aims	1
Background	1
2 MATERIALS AND GENERAL METHODS	8
Cell Culture	8
Cell Counts	8
Cell Viability	9
Clonogenic Assay	9
Transport of MGBG	9
Stability of MGBG	11
Protein Assay	12
Statistical Analysis	12
3 TRANSPORT OF MGBG AND ITS EFFECT ON SENSITIVE L1210 CELLS	13
Effects of MGBG on Growth, Viability and Clonogenicity	13
Characterization of the Initial Transport of MGBG	14
Intracellular Accumulation of MGBG During the First 20 Hours of Exposure	14
Kinetic Characterization of the Initial Transport Process	18
Inhibition of MGBG Uptake by Spermidine, Spermine and Putrescine	22
Effect of Extracellular pH on the Initial Uptake of MGBG	22

Efflux of MGBG 23
Countertransport of Unlabelled MGBG 23
Effect of Various Cations on the Uptake of MGBG 29
Sodium-dependency of MGBG uptake 29
Ability of various cations to substitute for sodium in uptake of MGBG 32
Effects of Ionophores on the Uptake of MGBG 38
Effect of Inhibitors of Energy Metabolism on the Uptake of MGBG 38
Intracellular Distribution of MGBG 45
Uptake of MGBG by Isolated Mitochondria 45
Selective Release of Cytosolic Constituents by Digitonin 46
Effect of Growth Rate on MGBG Transport 47

4 TRANSPORT OF MGBG AND DRUG RESISTANCE 54

Concentration of MGBG in L1210 Cells During 20 Days of Drug Exposure 54
Kinetic Characterization of MGBG Transport as Resistance Develops 58
Selection of L1210 Cells Highly Resistant to MGBG 62
Transport Properties of the Resistant Cells 66
Response of Multiple Drug Resistant (MDR) Cells to MGBG 66
Correlation between Transport and Resistance 73

5 DISCUSSION 77

6 SUMMARY AND FUTURE DIRECTIONS 86

REFERENCES 89

BIOGRAPHICAL SKETCH 94

LIST OF TABLES

Table		page
3-1	Comparison between the calculated and observed intracellular concentrations of MGBG during the first 20 hours of exposure	21
3-2	Comparison between the observed rates of MGBG uptake at different extracellular pH's with rates calculated on the basis of transport of MGBG $^{2+}$ only	26
3-3	Effects of various ions on MGBG uptake in L1210 cells	40
3-4	Effects of potassium and choline chloride on the sodium-dependent MGBG uptake in L1210 cells	41
3-5	Effects of ionophores on MGBG uptake in L1210 cells	42
3-6	Uptake of MGBG by isolated mitochondria	48
4-1	Effects of MGBG on the intracellular content of polyamines and putrescine in L1210 cells	57
4-2	Kinetics of the uptake of MGBG in untreated L1210 cells, cells that had "recovered" after exposure to MGBG, and a resistant subclone	65

LIST OF FIGURES

Figure		page
1-1	Structure of polyamines, related compounds and inhibitors of polyamine synthesis	7
3-1	Effects of various concentrations of MGBG on the proliferation of murine L1210 cells	15
3-2	Effects of various concentrations of MGBG on the viability of murine L1210 cells over time	16
3-3	Effect of MGBG on the clonogenicity of L1210 cells	17
3-4	Accumulation of MGBG by L1210 cells during the first 20 hours of exposure	19
3-5	Lineweaver-Burk plot of MGBG uptake	20
3-6	Competitive inhibition of MGBG uptake by spermidine, spermine and putrescine in control L1210 cells	24
3-7	Effects of medium pH on the initial uptake of MGBG	25
3-8	Efflux of MGBG	27
3-9	Effect of temperature on the efflux of MGBG	28
3-10	Effect of MGBG preloading on the uptake of labelled MGBG	30
3-11	Effect of medium concentration of MGBG on the efflux of labelled MGBG	31
3-12	Sodium-dependency of MGBG uptake in L1210 cells	33
3-13	Relationship between MGBG concentration and the rate of MGBG uptake in the presence or absence of sodium	34
3-14	Lineweaver-Burk plot of the sodium-dependent MGBG uptake in L1210 cells	35
3-15	The relationship between MGBG concentration and the rate	

of MGBG uptake in sodium chloride, choline chloride, and mannitol..... 36

3-16 Lineweaver-Burk plot of MGBG uptake in sodium chloride, choline chloride and mannitol..... 37

3-17 Effects of various ions on MGBG uptake in L1210 cells 39

3-18 Effects of inhibitors of energy metabolism on MGBG uptake.... 43

3-19 Effects of inhibitors of energy metabolism on MGBG uptake.... 44

3-20 MGBG remaining in the cell pellet after permeabilization of the plasma membrane by digitonin..... 49

3-21 Growth curve of L1210 cells..... 51

3-22 Viability of L1210 cells as a function of growth phase..... 52

3-23 Uptake of MGBG as a function of growth rate..... 53

4-1 Intracellular content of MGBG upon prolonged exposure to the drug..... 56

4-2 Influx of MGBG before and after the peak intracellular concentration of drug had been attained..... 59

4-3 Efflux of MGBG before and after the peak intracellular concentration of drug had been attained..... 60

4-4 Influx, efflux and apparent steady-state intracellular content of MGBG on day 6 of exposure to drug..... 61

4-5 Competitive inhibition of MGBG uptake by spermidine, spermine and putrescine in L1210 cells exposed to MGBG for 3 weeks..... 63

4-6 Uptake of MGBG in the "recovered" L1210 cells..... 64

4-7 Effects of various concentrations of MGBG on growth of MGBG-resistant L1210 cells..... 67

4-8 Effects of various concentrations of MGBG on viability of MGBG-resistant L1210 cells..... 68

4-9 Effects of various concentrations of MGBG on growth of MGBG-resistant L1210 cells after one month of drug-free period..... 69

4-10 Effects of various concentrations of MGBG on viability of

MGBG-resistant L1210 cells after one month of drug-free period..... 70

4-11 Accumulation of MGBG in the MGBG-resistant L1210 cells..... 71

4-12 Competitive inhibition of MGBG uptake by spermidine, spermine and putrescine in MGBG-resistant L1210 cells..... 72

4-13 Comparison of MGBG dose-response curve of parental drug-sensitive ($AuxB_1$) and multidrug-resistant (CH^RC_5) CHO cell lines..... 74

4-14 Correlation between intracellular content of MGBG and the rate of cell proliferation in various type of L1210 cells..... 76

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When murine leukemia L1210 cells were exposed to methylglyoxal bis[guanylhydrazone] (MGBG), drug concentrations less than 0.3 μM had no effect on cell proliferation, and concentrations greater than 5 μM were lethal except in subclones of cells selected for resistance. Intermediate concentrations decreased the rate of cell growth temporarily. L1210 cells accumulated MGBG avidly, and this accumulation was crucial for cytotoxicity and the development of resistance. My studies focused on the transport process, and most were conducted with 0.3 - 5 μM MGBG. With 1 μM MGBG in the medium, the apparent intracellular concentration of MGBG reached approximately 2000 μM in 24 hours. Transport of MGBG seemed to be energy dependent and carrier-mediated with saturable kinetics characterized by a V_{max} and K_m in the presence of sodium, of 296 ± 19 pmole/10^6 cells/hour and 4.72 ±

0.169 μM, respectively. The rate of transport depended on temperature, extracellular pH and cell proliferation. Inhibitors of energy metabolism, ionophores which dissipate the plasma membrane electrochemical gradient, and the replacement of sodium ions by other cations, all significantly decreased the rate of accumulation of MGBG. Naturally-occurring polyamines, spermidine and spermine, and putrescine competed with MGBG for uptake.

Within 48 hours of exposure to 1 μM MGBG, the rate of growth of L1210 cells had decreased substantially. Despite the continued presence of MGBG in the medium, the intracellular concentration of MGBG decreased to approximately 500 μM by day 6 and even further to 100 - 400 μM well in advance of the full recovery of cell proliferation by 2 weeks. The development of resistance during this time was due to a decrease in MGBG influx; the rate of efflux remained unchanged. In addition, an MGBG-resistant subclone of cells also exhibited a deficiency in MGBG transport. In both types of resistant cells, as well as in previously untreated cells, the relative but not absolute affinity of the transporter for MGBG, spermidine, spermine and putrescine were constant. In all cells, an apparent intracellular concentration of MGBG greater than 400 μM was associated with toxicity.

Resistance to MGBG was not associated with multiple drug resistance or expression of P-glycoprotein.

CHAPTER 1

INTRODUCTION

Specific Aims

Methylglyoxal bis[guanylhydrazone] (MGBG) has been used clinically in the treatment of cancer for several years. MGBG accumulates quite extensively in sensitive targets like mouse leukemia L1210 cells. I have observed that even a relatively small decrease in the extent of accumulation of MGBG results in resistance to the antiproliferative and cytotoxic actions of the drug. My dissertation focuses on the transport of MGBG. In general terms, I propose to

1. characterize the transport and accumulation of MGBG in sensitive (previously untreated) L1210 cells.

2. define the mechanism(s) of resistance to MGBG that develops quickly (days) when L1210 cells are exposed to sublethal concentrations of MGBG.

3. define the mechanism(s) of resistance to MGBG in a clone of L1210 cells selected for resistance to MGBG (resistance persists even when the cells are grown in drug-free medium).

Background

The structure of MGBG is depicted in Figure 1-1. The figure also includes structures of putrescine, spermidine, spermidine, N-acetylspermidine and alpha-difluoromethyl ornithine (DFMO), a well-known inhibitor of ornithine decarboxylase. The bis[guanylhydrazone] represent a chemical family of compounds in which terminal amidine groups are separated by variable aromatic or aliphatic structures frequently containing interposed nitrogen

groups. Although a number of these compounds posses significant antitumor activity, they differ substantially in their mode of action and in their ability to interfere with various biochemical pathways. For example, the antileukemic action of the aromatic bis[guanylhydrazone], of which 4,4'-diacetyldiphenylurea bis[guanylhydrazone] is probably the best studied example, has been correlated with the ability of these compounds to bind to calf thymus DNA and to inhibit DNA-dependent DNA polymerase (Dave et al., 1977). The mode of action of the aliphatic compounds is much less understood. Unlike the aromatic bis[guanylhydrazone], MGBG and other aliphatic analogs bind weakly to DNA and do not inhibit the activity of DNA polymerase (Dave et al., 1977).

Early clinical trials revealed that MGBG had very impressive antiproliferative activity, particularly against acute myelocytic leukemia (Carbone et al., 1964, Freireich, et al., 1962, Levin et al., 1965, Porter et al., 1979, and Regelson and Holland, 1963). At optimal dose levels (150 mg/m^2 daily), MGBG alone produced complete remission in about 45% of patients (Levin et al., 1965). This drug can also be used in combination with other chemotherapeutic drugs, such as 6-mercaptopurine, and remission rates of 35 - 45% have been reported among leukemia patient (Boiron et al., 1965 and Weil et al., 1969). MGBG was also found to be effective in the treatment of some solid tumors, including Hodgkin's disease (46% responsive) and non-Hodgkin's lymphoma (37% responsive). One of the interesting approaches in the use of MGBG was its sequential administration after DFMO (alpha-difluoromethyl ornithine), an inhibitor of polyamine biosynthesis.

Despite being introduced into clinical use as an anticancer agent about 30 years ago, the molecular basis for MGBG's activity remains obscure. Until recently, the mechanism of action of MGBG was thought to be related to its various relationships with the biological polyamines. MGBG has some similarity

to spermidine (Hamilton and La Placa, 1968), competes with spermidine and spermine for uptake into the cell (Dave and Caballes, 1973, Porter et al., 1981 and 1982), strongly inhibits the polyamine biosynthetic enzyme, S-adenosylmethionine decarboxylase (Holtta et al., 1973, Williams-Ashman and Schenone, 1972) and inhibits the polyamine degradative enzyme, diamine oxidase (Holtta et al., 1973). It was the inhibition of S-adenosylmethionine decarboxylase by MGBG that was thought initially to be responsible for the antiproliferative activity of the drug. In cells treated with MGBG, the concentration of putrescine increases whereas spermidine and spermine decrease slowly as a consequence of pathway blockade. The observation that spermidine prevented the antiproliferative effects of MGBG (Mihich, 1965), led initially to the conclusion that this effect on the naturally-occurring polyamines was important. The action of spermidine is now attributed more to competition for cellular uptake than to replenishment of polyamine pools. A number of laboratories (Holtta et al., 1979, 1981, Newton and Abdel-Monem, 1977, Pleshkewych et al., 1982, Seppanen et al., 1980) have been unable to correlate these changes in polyamine pool size with inhibition of cell growth. Moreover, DFMO, a highly specific inhibitor of polyamine biosynthesis, is cytostatic (Mamont et al., 1978), whereas MGBG is cytotoxic, a finding that suggests different mechanisms of action for the two drugs.

On the basis of considerable ultrastructural and biochemical evidence, there is now sufficient cause to believe that the mitochondrion may, in fact, be the target responsible for the antiproliferative activity of MGBG. Indeed, 4, 4'-diacetyldiphenylurea-bis[guanylhydrazone], an aromatic bis[guanylhydrazone] with potent antiproliferative activity, causes profound ultrastructural damage to mitochondria (Mikles-Robertson et al., 1981) but has no effect on polyamine biosynthesis (Corti et al., 1974). Ultrastructural studies in a variety of cell types

treated in vitro (Mikles-Robertson et al., 1979, Pathak et al., 1977, and Wiseman et al., 1980) or in vivo (Pleshkewych et al., 1980 and Porter et al., 1979) with MGBG reveal that mitochondria were selectively and significantly damaged.

In addition, the mitochondria were functionally impaired by MGBG. The metabolic effects of MGBG include 1) decreased rates of oxidation of pyruvate (Pleshkewych et al., 1980) and long chain fatty acids (Nikula et al., 1984 and Brady et al., 1987) ; 2) a decrease in the intracellular concentration of ATP with concommitant increases in ADP concentration (Pine and DiPaolo, 1966 and Regelson and Holland, 1963) and lactate production (Porter et al., 1982) ; and 3) a decreased rate of incorporation of acetate into lipid (Pine and DiPaolo, 1966).

A study of the effects of MGBG on isolated rat liver mitochondria (Byczkowski et al., 1981) demonstrated that at drug concentrations comparable to those attained intracellularly, MGBG significantly inhibits state 4 respiration, but has less of an effect on either state 3 or uncoupled respiration. Byczkowski et al. speculated that this may be due to the fact that in the absence of ADP (state 4), mitochondria generate a significant electrochemical gradient across their inner membrane. Since MGBG is a cation under physiological conditions, it might accumulate at or within the inner membrane because of its negative potential. Similar selective binding characteristics have been noted for other cationic compounds. Rhodamine dyes that are positively charged stain mitochondria specifically, whereas uncharged rhodamines and the negatively charged dye, fluorescein, do not. It is possible that MGBG neutralizes the net negative surface potential of the mitochondrial inner membrane, perhaps by binding to phospholipids. This might influence cation binding and/or transport and secondarily affect mitochondrial bioenergetics (Toninello et al., 1988). In any

case, the exact mechanism by which MGBG influences mitochondrial metabolism is unknown. It is likewise not known whether or not these actions play a role in the effects of MGBG on mitochondrial DNA replication described below.

MGBG inhibits the replication of mitochondria DNA (mtDNA) at concentrations of drug that do not seem to interfere with the replication of nuclear DNA (Feuerstein et al., 1979). Drug-induced inhibition of mtDNA replication is likely to occur shortly after exposure to MGBG (Nass, 1984). Our laboratory has observed that the decrease in cellular content of mtDNA with time can be accounted for by lack of replication coupled with dilution of the residual genome upon repetitive cellular division (Bortell, 1987). These results were obtained in our laboratory with use of dot-blot hybridization methods involving whole cell lysates and ^{35}S labelled full-length mouse mtDNA probe. Although we know little about the mechanism(s) by which MGBG adversely affects the ultrastructure, bioenergetics and replication of mitochondria, it does seem likely that these actions relate to the antiproliferative and cytotoxic effects of the drug and many of its congeners.

MGBG seems to accumulate in dividing cells by utilizing the facilitated carrier mechanism for spermidine (Dave and Caballes, 1973, Porter et al., 1981). Seppanen et al. (1981) found that MGBG uptake is critically dependent on the growth rate of tumor cells (i.e., slowly dividing cells transport less MGBG than rapidly dividing cells). Moreover, Mikles-Robertson et al. (1979) found that MGBG cytotoxicity also correlates with growth rate. Athough several descriptions of the transport of MGBG have been published, the process is still poorly defined. MGBG uptake is thought to involve an energy dependent saturable carrier (Porter et al., 1982; Field et al., 1964), in that cells concentrate the drug, and concentration gradients across the plasma membrane as high as

1000-fold have been reported. Polyamine and/or MGBG transport has been studied in different cell lines and tissues including neuroblastoma (Rinehart and Chen, 1984), fibroblast (Pohjanpelto, 1976), Ehrlich ascites carcinoma (Seppanen et al., 1980,1981,1982 ; Seppanen, 1981), mammary gland explants (Kano and Oka, 1976), slices of rat lung and other tissues (Gordonsmith et al., 1985; Smith and Wyatt, 1981), and rat lung perfused in situ (Reynolds et al., 1985). In general, it appears that putrescine, spermidine, spermine, and MGBG are transported by common membrane transport systems which are distinguishable from other defined systems (e.g., for various amino acids). The transport is saturable and temperature-dependent, with maximal rates evident at 37°C. MGBG transport is inhibited by uncouplers of oxidative phosphorylation and certain respiratory poisons, dependent on the proliferative status of the cells and, in some instances, subject to hormonal regulation. Whether polyamine/MGBG transport actually occurs via the same mechanisms in all types of cells in any particular organism is also unclear. The development of more efficacious drugs in this class of compounds seems to depend on further understanding of the mechanism of transport, and, perhaps more importantly, how the transport process is regulated.

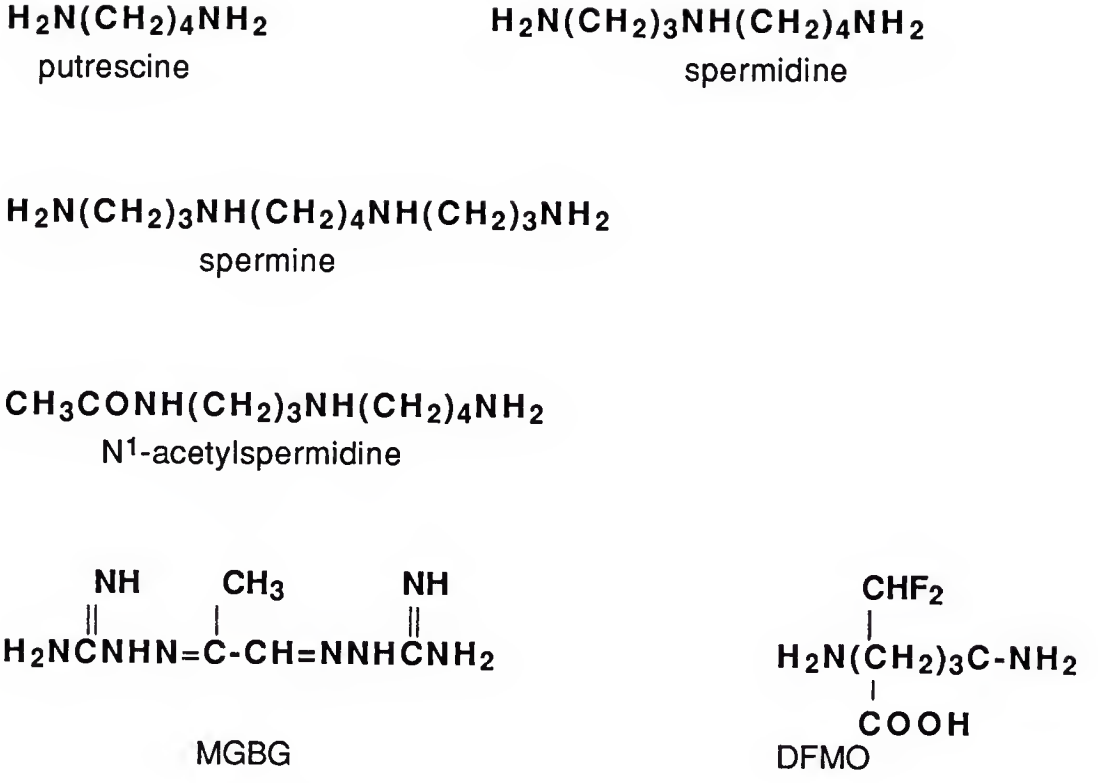

FIGURE 1-1 : Structure of polyamines, related compounds and inhibitors of polyamine synthesis.

CHAPTER 2

MATERIALS AND GENERAL METHODS

Cell Culture

Murine L1210 cells (obtained from American Type Culture Collection, Rockville, Maryland) were allowed to grow in suspension in 25 or 75 cm^2 canted-neck tissue culture flasks (Fisher Scientific) in an incubator (National Appliance Company, Portland, Oregon) in an atmosphere of air : CO_2 (95 : 5, v/v) at 37°C. Cells were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium (Cellgro^R, GIBCO Laboratories) supplemented with 10 % heat-inactivated horse serum (GIBCO Laboratories), 16 mM HEPES (3-[N-morpholino] propanesulphonic acid) and 8 mM MOPS (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid), at a final pH of 7.40. All chemicals were obtained from Sigma Chemical Co. unless otherwise specified. Cells were maintained in logarithmic growth by reseeding an aliquot of cells into fresh medium every two days to a starting concentration of 7×10^4 cells per ml.

Cell Counts

Aliquots (100 or 200 µl) of cells were diluted in 10 ml of Hematall diluent (Fisher Scientific). Cell number was determined by electronic particle analysis (Coulter Counter, Model ZF, Coulter Electronics, Hialeah, FL). Two aliquots were counted from each flask, and the mean was determined. Because cells were reseeded every two days, a calculation was devised to plot cell accumulation as a single, continuous line over the the entire period of an experiment. Cumulative counts were calculated as:

$$\text{Cumulative count}(n+1) = \text{Cumulative count}(n) \times \frac{\text{conc. of viable cells } (n+1)}{\text{conc. of viable cells } (n)}$$

The population doubling time of the cells was determined as follows: $N = N_0 e^{+kt}$; where t = time, N_0 is the number of cells present at t = 0, k = ln 2/ doubling time, and the doubling time is the value of t when $N = 2N_0$.

Cell Viability

Trypan blue dye (Eastman Kodak, Rochester, NY) at a final concentration of 0.06% was added to 100 µl aliquots of cells. The cells were mixed well, and a 10 µl aliquot was transferred to a hemacytometer (Reichert Scientific Instruments, Buffalo, NY). At least 100 cells per sample were examined by phase contrast microscopy (Ernst Leitz, Wetzlar, Germany) at 200X magnification. Percent viability of cells was determined as follows:

$$\% \text{ viability} = \frac{\text{number of cells excluding trypan blue dye x 100}}{\text{total number of cells}}$$

Clonogenic Assay

Colony-forming ability of L1210 cells was determined by growth in soft agar (Chu and Fischer, 1968). Pre-autoclaved Agar Noble (DIFCO Laboratories, Detroit, MI) was admixed at 37°C with RPMI 1640 medium containing 20% heat-inactivated horse serum to achieve 1mg agar/ml final concentration . A 4 ml aliquot of the prepared medium was transferred into each cloning tube. L1210 cells from selected experitments were counted and resuspended into cloning tubes over the concentration range of 100 to 10,000 cells per tube. Cloning tubes were cooled down at 0 - 4°C for 15 minutes prior to incubation at 37°C. Colony counts were determined 2 weeks after the start of incubation.

Transport of MGBG

The assay of MGBG influx and efflux was based on the use of methylglyoxal-bis[^{14}C]guanylhydrazone ([^{14}C] MGBG) (specific activity 92.8 µCi/mg or 25.6

mCi/mmole, Amersham). A stock solution of 1 mM [^{14}C] MGBG was prepared aseptically. Aliquots of this stock solution were added to the incubation medium to obtain the indicated final concentration of drug.

Murine L1210 cells were exposed to various concentrations of [^{14}C] MGBG for the indicated times at 37°C and pH 7.40 unless otherwise indicated. Incubations were terminated by centrifugation. One-ml aliquots of cell suspensions were centrifuged at 15,000g for 10 seconds in Eppendorf tubes. The supernatant was discarded, and the cell pellet was washed twice by centrifugation with one ml of ice-cold medium containing 1 mM unlabelled MGBG. The tip of each Eppendorf tube, which contained the cell pellet, was cut off and transferred to a scintillation vial containing 10 ml of Liquiscint^{R} (National Diagnostics, Manville, NJ) for quantitation by liquid scintillation counting in Beckman model LS 7000.

Cell counts and viability were obtained with use of a hemacytometer. The diameter and volume of cells were determined by the method of Schwartz et al. (1983). Uniform polymeric microspheres (Polyscience, Warrington, PA) from 4.72 to 10.0 μ in diameter were diluted in Hematall, and the particle size measured electronically with a FACS Analyzer (Becton-Dickinson, Sunnyvale, CA) with the amplifier in the log mode. The peak channel number was plotted against the corresponding diameter and volume for each size of calibrated microbead to obtain a standard curve.

Aliquots of $1x10^6$ cells were collected, pelleted, and resuspended in 0.5 ml Hematall for analysis. The peak channel number for the cells was plotted on the calibration curve to obtain the approximate cell diameter and volume. The diameter of drug-naive L1210 cells was 10.6 μ and cell volume was 5.68 μl per 10^6 cells. There was no significant change in the size of L1210 cells during or after an exposure to MGBG when the cells were observed under a light

microscope in the presence of standard beads. The apparent intracellular concentration of [^{14}C] MGBG (μM) in all experiments was calculated assuming a volume of 5.68 μl per 10^6 cells. In studies of efflux, cells were preincubated in [^{14}C] MGBG as indicated in subsequent chapters.

Preliminary experiments had revealed that 1) the rate of accumulation of MGBG by cells was constant for at least 20 minutes; and 2) although 1 mM unlabelled MGBG was routinely included in the wash of cells, its presence did not make much difference.

Stability of [^{14}C] MGBG

It is generally believed that MGBG does not undergo biotransformation in higher animals (Warrell and Burchenal, 1983), in part because no metabolites of MGBG have been detected in urine, feces and various tissues and no radioactive carbon dioxide was expired after in vivo administration of [^{14}C] MGBG. Nonetheless, we further established the stability of [^{14}C] MGBG upon incubation with L1210 cells by thin layer chromatography. L1210 cells were exposed to [^{14}C] MGBG for 24 and 48 hours. Aliquots of cell suspension were taken, centrifuged and the supernatant was then removed. The cell pellet was washed twice at 0 - 4°C with media containing 1 mM unlabelled MGBG, then resuspended in deionized water to lyse the cells. Trichloroacetic acid was added to a final concentration of 10% and the precipitated protein was removed by centrifugation. Aliquots of supernatant were spotted on precoated thin layer plates of Silica Gel 60 F254 (E. Merck, Darmstadt, Germany). Chromatograms were developed with 50% n-butanol, 30% water and 20% glacial acetic acid. Unlabelled MGBG was located by fluorescence quenching, and its Rf was found to be 0.49 - 0.53. [^{14}C] MGBG was located by autoradiography with use of Kodak SB Film, and its migration was indistinguishable from that of authentic MGBG. The only radioactivity observed after 24 and 48 hours of incubation of

[^{14}C] MGBG with L1210 cells was that of the drug itself. This observation confirmed that [^{14}C] MGBG was stable and was not metabolized by the cells.

Protein Assay

Protein was measured by the method of Lowry et al. (1951) with use of bovine serum albumin as standard.

Statistical Analysis

The two-tailed Student t-test was used to analyze comparisons; the significance level was set at $P < 0.05$. Lineweaver-Burk graphs were evaluated by least squares regression analysis.

CHAPTER 3

TRANSPORT OF MGBG AND ITS EFFECT ON SENSITIVE L1210 CELLS

This chapter deals with 1) the effects of varying doses of MGBG on the proliferation, viability and clonogenicity of L1210 cells; 2) the characterization of the processes involved in the transport of MGBG into and out of these cells upon first exposure to the drug; 3) an assessment of the intracellular distribution of the MGBG, especially with regard to possible accumulation within mitochondria; and 4) the relationship between transport of MGBG and the rate of cell growth.

Effects of MGBG on Growth, Viability and Clonogenicity

To determine the effects of MGBG on cell growth and viability, L1210 cells in logarithmic growth were incubated continuously in 0 to 20 μM drug at 37°C, pH 7.40. Cells were reseeded every two days in fresh medium containing MGBG. Cell count and viability were determined daily. The cumulative count of viable cells is depicted in Figure 3-1, and the percent of total cells that were viable is presented in Figure 3-2. Untreated cells exhibit a doubling time of approximately 9 hours.

MGBG at concentrations as high as 20 μM had minimal effect on cell growth or viability for the first few days of exposure. Our laboratory (Bortell, 1987) has reported that the cellular content of mt DNA decreases markedly during this time. The decrease was found to reflect dilution of the mitochondrial DNA due to lack of its replication coupled with continuing cell division. Thereafter cell

growth was inhibited by MBGB in a dose-dependent manner, perhaps because of the depletion of mt DNA. At MGBG concentrations between 0.3 and 1.0 μM, the growth rates were markedly inhibited for about 10 days after which recovery to rates virtually identical to untreated cells was observed. At these doses, viability was also adversely affected in a concentration-dependent manner, but it did not fall below 60%. Viability recovered to the control value of nearly 100% when growth rate recovered. At the intermediate dose of 5.0 μM MGBG, the percent of viable cells decreased to a plateau of about 40%, and recovery of growth had not occurred by day 17 of treatment. The cytotoxic affect of MGBG was apparent when the medium contained 20 μM MGBG. Cell viability decreased progressively, and no living cells could be detected after day eight.

Further experiments were performed to determine the effects of MGBG on clonogenicity of murine L1210 cells in soft agar. L1210 cells were pretreated with different concentrations of MGBG (0 - 10 μM) for 24 or 72 hours before reseeding in drug-free RPMI 1640 medium containing 1 gm/ml Agar Noble. MGBG inhibited the clonogenicity of L1210 cells in soft agar in a concentration-dependent manner (Fig 3-3). The inhibitory effect of MGBG on the clonogenicity of L1210 cells was evident as early as 24 hours after MGBG exposure, whereas the cell viability was unaffected at that time.

Characterization of the Initial Transport of MGBG

Intracellular Accumulation of MGBG During the First 20 Hours of Exposure

Although we have measured the intracellular content of MGBG over a few weeks of incubation (see below), this portion of the proposal focuses on the first 20 hours of incubation. It is during this time that the L1210 cells accumulated large amounts of MGBG. By 20 hours, the replication of mitochondrial DNA has been inhibited, but the rate of cell growth and cell viability have remained unaffected.

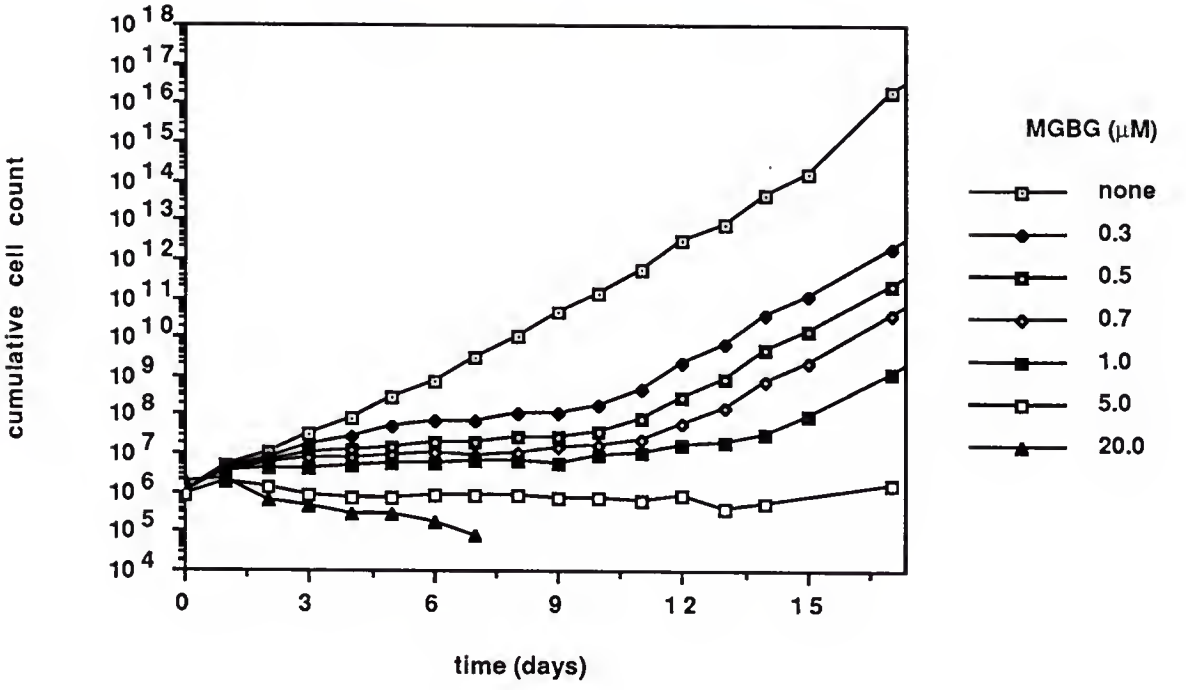

FIGURE 3-1 : Effects of various concentrations of MGBG on the proliferation of murine L1210 cells. Cells in logarithmic growth were incubated continuously in 0 - 20 μM MGBG and were reseeded every two days in fresh medium containing MGBG. Cell counts were determined by electronic particle analysis (Coulter Counter). Cumulative cell counts were calculated and cell accumulation was plotted as a single continuous line over the entire period of an experiment.

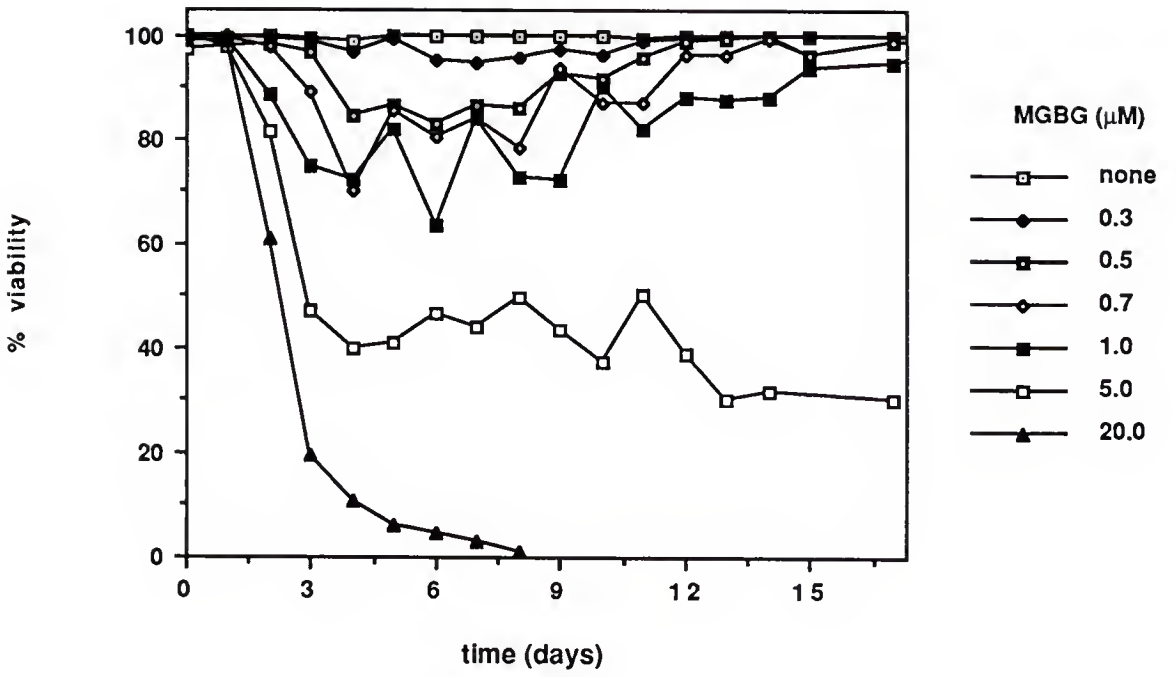

FIGURE 3-2 : Effects of various concentrations of MGBG on the viability of murine L1210 cells over time. Cells in logarithmic growth were incubated continuously in 0 - 20 μM MGBG and were reseeded every two days in fresh medium containing MGBG. Cell viability was determined with use of the trypan blue dye exclusion method.

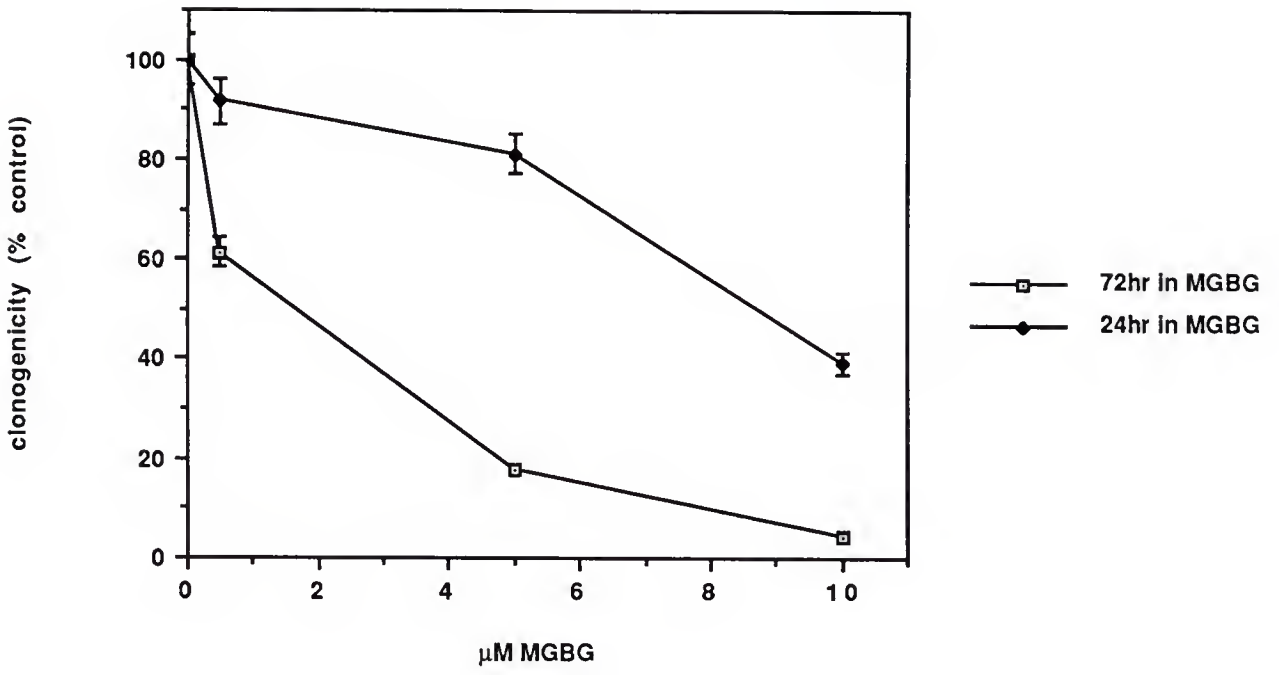

FIGURE 3-3 : Effects of MGBG on the clonogenicity of L1210 cells. L1210 cells were exposed to different concentrations of MGBG (0 - 10 µM) for 24 or 72 hours prior to reseeding in RPMI 1640 medium containing 1 mg/ml Agar Noble. Clonogenicity was determined by an ability of cells to form colonies in agar after 2 weeks of incubation, and values are expressed as percent control.

Cells in logarithmic growth were incubated in various concentrations of [^{14}C] MGBG at 37° C, pH 7.40. At various times, aliquots of cells were harvested and washed at 0 °C by centrifugation. Although the routine washing procedure involves the use of unlabelled 1 mM MGBG, initial experiments revealed that little added radiolabel was displaced from the cells by the carrier. In addition, the rate of efflux of radiolabel from the cells is sufficiently slow even at 37°C to permit this approach to separation of cells from incubation medium. The results are presented in Figure 3-4. MGBG accumulated within cells at reasonably constant rates dependending on the initial extracellular MGBG concentration. It is apparent in Figure 3-4 that the apparent intracellular concentration of MGBG exceeded that of the medium by about 1000-fold within 12 hours.

Kinetic Characterization of the Initial Uptake Process

The initial uptake of MGBG occurs by a saturable process. To determine K_m and V_{max}, L1210 cells in logarithmic growth were reseeded at a density of 3 x 10^5 cells per ml in prewarmed (37°C) media containing different concentrations of [^{14}C] MGBG at pH 7.40. "Intracellular" radioactivity was determined 20 minutes later (see Chapter 2 for details of centrifugation and washing of the cells). As noted above, all of the radioactivity, even 48 hours after the start of incubation, remained stable as [^{14}C] MGBG. The amount of drug in cells was expressed as pmole per 10^6 cells. Four separate experiments were conducted. Lineweaver-Burk plots were constructed (Figure 3-5), and K_m and V_{max} were determined by linear regression. The K_m and V_{max} for uptake of MGBG at 37°C were 4.72 ± 0.17 μM and 296 ± 19 pmole/10^6 cells/hour, respectively.

Interestingly, the amount of intracellular [^{14}C] MGBG over the first 20 hours of exposure to the drug (Figure 3-4) could be predicted with use of the kinetic parameters determined over 20 minutes (Table 3-1). Given the volumes of cells

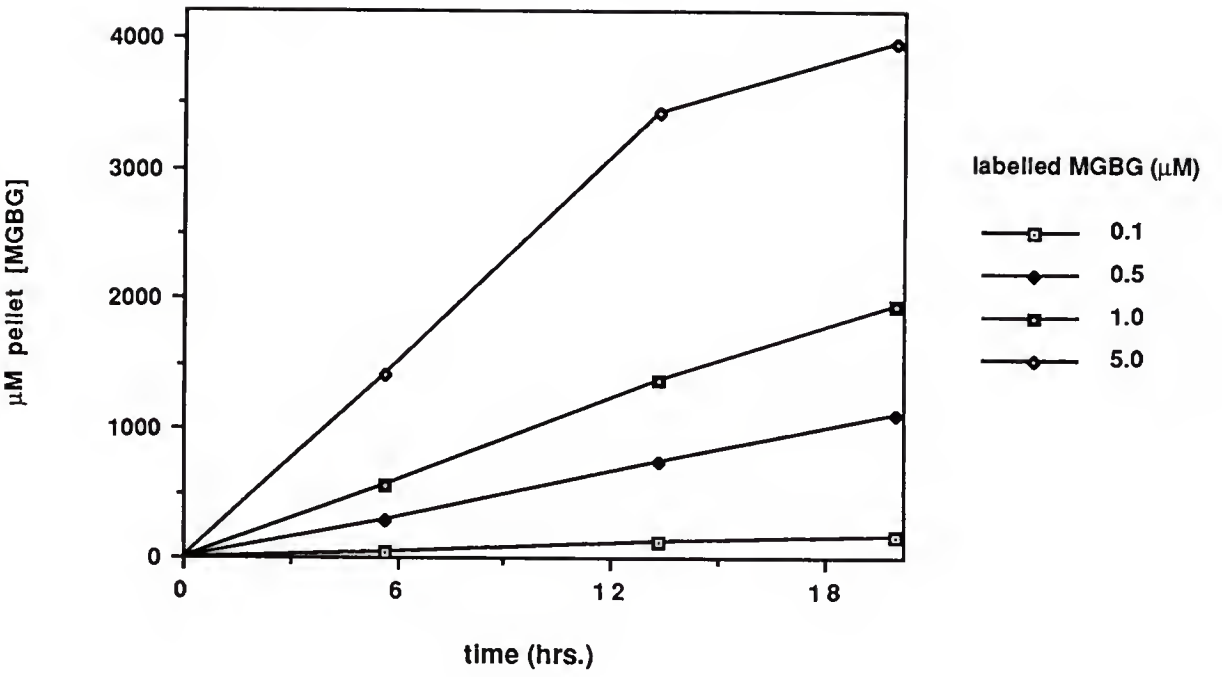

FIGURE 3-4 : Accumulation of MGBG in L1210 cells during the first 20 hours of exposure. Cells in logarithmic growth were incubated in 0.1 - 5 µM [^{14}C] MGBG. At various times, aliquots of cells were harvested and cell pellet was washed twice by centrifugation with use of medium containing 1 mM MGBG. Cell counts and viability were obtained with use of a hemacytometer and the results were expressed as apparent intracellular concentrations of [^{14}C] MGBG (µM).

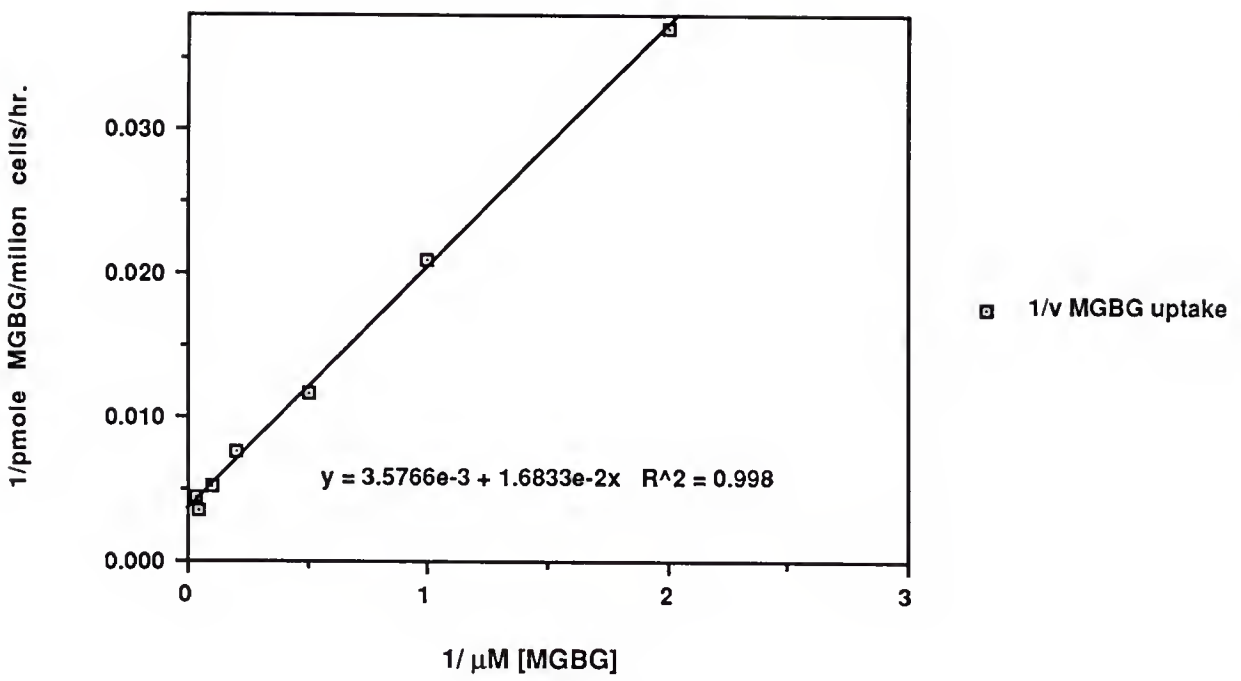

FIGURE 3-5 : Lineweaver-Burk plot of MGBG uptake in control L1210 cells. L1210 cells in logarithmic growth were reseeded at a density of 3 x 10^5 cells per ml in media containing different concentrations of [^{14}C] MGBG. Intracellular radioactivity was determined 20 minutes later and the amount of drug in cells was expressed as pmole [^{14}C] MGBG per 10^6 cells. The K_m and V_{max} were determined by linear regression of the Lineweaver-Burk Plot.

TABLE 3-1 : Comparison between the calculated and observed intracellular concentrations of MGBG during the first 20 hours of exposure.

hrs. after incubation	Intracellular concentration of [^{14}C] MGBG (μM)							
	0.1 μM		0.5 μM		0.93 μM		4.8 μM	
	calculated	observed	calculated	observed	calculated	observed	calculated	observed
5.7	60	38.9 (1.5)	271.0	289.8 (11.3)	487.8	561.5 (34.5)	1488.9	1420.5 (49.1)
13.3	141.3	131.7 (9.6)	637.6	735.0 (32.9)	1147.6	1374.9 (79.5)	3503.1	3423.5 (224)
20.1	169.5	164.8 (10.1)	960.8	1110 (120)	1729.2	1946.5 (136)	5074.5	3944.5 (263)

Note : The intracellular concentration of MGBG at various times was calculated with use of Michaelis-Menten Equation ($v = V_{max}[S]/K_m + [S]$), using the predetermined values for the V_{max} and K_m of 296 $\pm$ 19 pmole/10^6 cells/hour and 4.72 $\pm$0.169 μM, respectively. Efflux was ignored in the calculations. Each observed value represents the mean $\pm$ SEM.

and of the incubation media, the change in extracellular concentrations of MGBG over 20 hours did not exceed 25% of the initial concentration. Indeed, except for the lowest initial concentration of drug, the decrease in extracellular [MGBG] was considerably less than 25%.

Experiments were also conducted at 0 - 4°C. Rates of uptake of 10 μM MGBG at 0 - 4°C was less than 10% of that seen at 37°C. This result would be anticipated if the uptake of MGBG depended on active transport.

Inhibition of MGBG Uptake by Spermidine, Spermine and Putrescine

L1210 cells were incubated in medium containing different concentrations of [^{14}C] MGBG (1.2 - 58.6 μM) with or without 10 μM spermidine, spermine or putrescine. The incubation was carried out for 20 minutes at 37 °C. Uptake of MGBG was inhibited competitively by spermidine, spermine and putrescine (Figure 3-6). This result implies that MGBG and the naturally occurring polyamines, spermidine, spermine and putrescine, share a common transport mechanism. The K_i's of spermidine, spermine and putrescine for the inhibition of MGBG uptake were 0.524 ± 0.03, 0.582 ± 0.029 and 3.74 ± 0.029 μM, respectively.

Effect of Extracellular pH on the Initial Uptake of MGBG

Logarithmically-growing L1210 cells were exposed to [^{14}C] MGBG (2.0 μM) for 0 to 10 minutes at 37°C over the range of extracellular pH between 7.0 and 7.7. The rates of uptake of MGBG were strongly influenced by pH . An increase in extracellular hydrogen ion concentration enhanced the rate of uptake of [^{14}C] MGBG. Similar results were observed when the experiments were conducted for 0 to 75 minutes with 0.8 and 8.5 μM [^{14}C] MGBG (Figure 3-7). MGBG is a weak base with pK_a's of about 7.5 and 9.2 at 25°C (Williams-Ashman and Seidenfeld, 1986). At physiological pH, MGBG exists primarily as a mixture of monovalent and divalent cations. The results depicted in Figure 3-7 suggest

that it is the divalent form of MGBG (MGBG $^{2+}$) that is transported into L1210 cells. Some evidence for this idea derived from consideration of the competitive inhibition of MGBG transport by the naturally occurring polyamines. For example, spermidine, the extended molecular conformation of which is quite similar to that of MGBG, has pK_a values of about 8.4, 9.8 and 10.8 at 25°C (Williams-Ashman and Seidenfeld, 1986), and exists almost exclusively as a mixture of divalent and trivalent cations at physiological pH. The hypothesis that $MGBG^{2+}$ is the substrate for transport is supported further by the good agreement between the observed and calculated values of MGBG uptake presented in Table 3-2. The calculations were based on Henderson-Hasselbach computations of the concentrations of $MGBG^{2+}$ at different pH's and on the assumption that only $MGBG^{2+}$ is transported.

Efflux of MGBG

The following experiments were designed to study the characteristics of efflux of MGBG from L1210 cells. Logarithmically-growing L1210 cells were exposed to 1.5 μM [^{14}C] MGBG for four hours before centrifugation and resuspension in drug-free medium. The apparent intracellular concentration of MGBG was about 400 μM at the time of resuspension. The efflux of radioactivity with time is depicted in Figure 3-8. Efflux occurs by a process with approximately first-order characteristics with a half-life of about three hours. Experiments were also conducted at 0 - 4°C. The results reveal that the efflux of MGBG at this low temperature was minimal (Figure 3-9), which would be anticipated if the efflux of MGBG depended on active transport.

Countertransport of Unlabelled MGBG

To explore the possibility that MGBG transport is mediated by an antiport carrier, the effect of preloading the cells with unlabelled MGBG on the subsequent rate of uptake of [^{14}C] MGBG was assessed. L1210 cells were

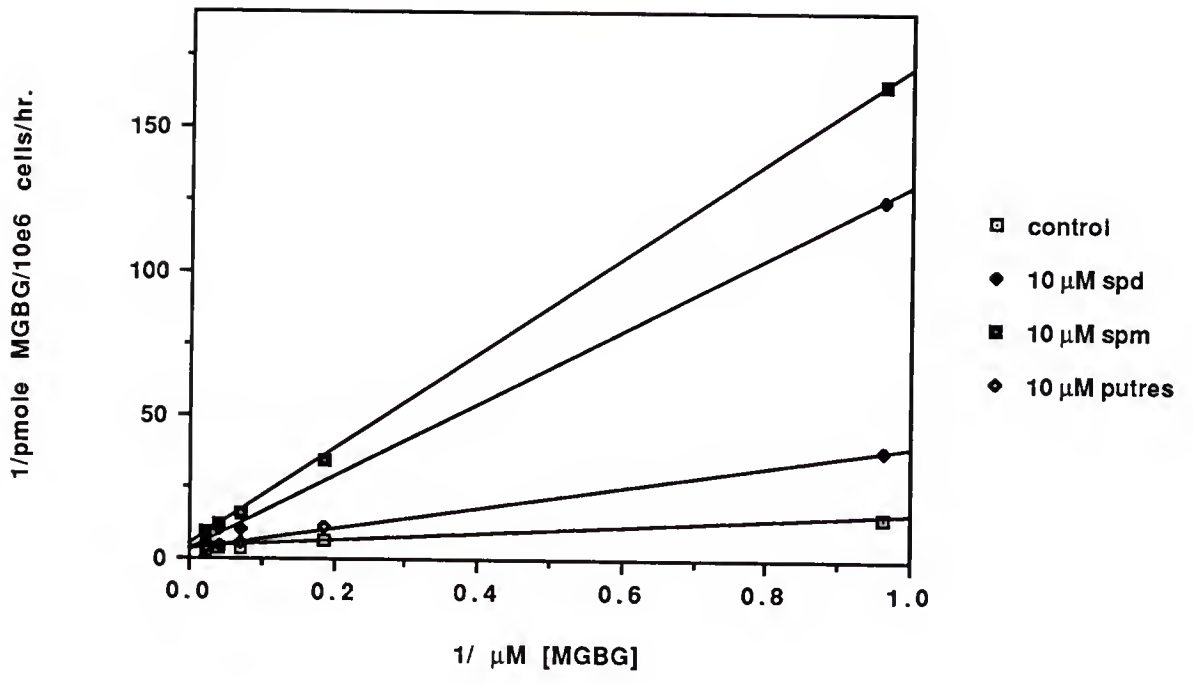

FIGURE 3-6 : Competitive inhibition of MGBG uptake by spermidine, spermine and putrescine in control L1210 cells. Control L1210 cells in logarithmic growth were reseeded at a density of 3 x 10^5 cells per ml in media containing different concentrations of [^{14}C] MGBG (1.7 - 80.6 μM) in the presence or absence of 10 μM spermidine (spd), spermine (spm) or putrescine (putres). The uptake of [^{14}C] MGBG at 37°C was assessed after 20 minutes incubation. The K_i's of spermidine, spermine and putrescine for the uptake of MGBG were 0.524 ± 0.03, 0.582 ± 0.029 and 3.74 ± 0.029 μM, respectively.

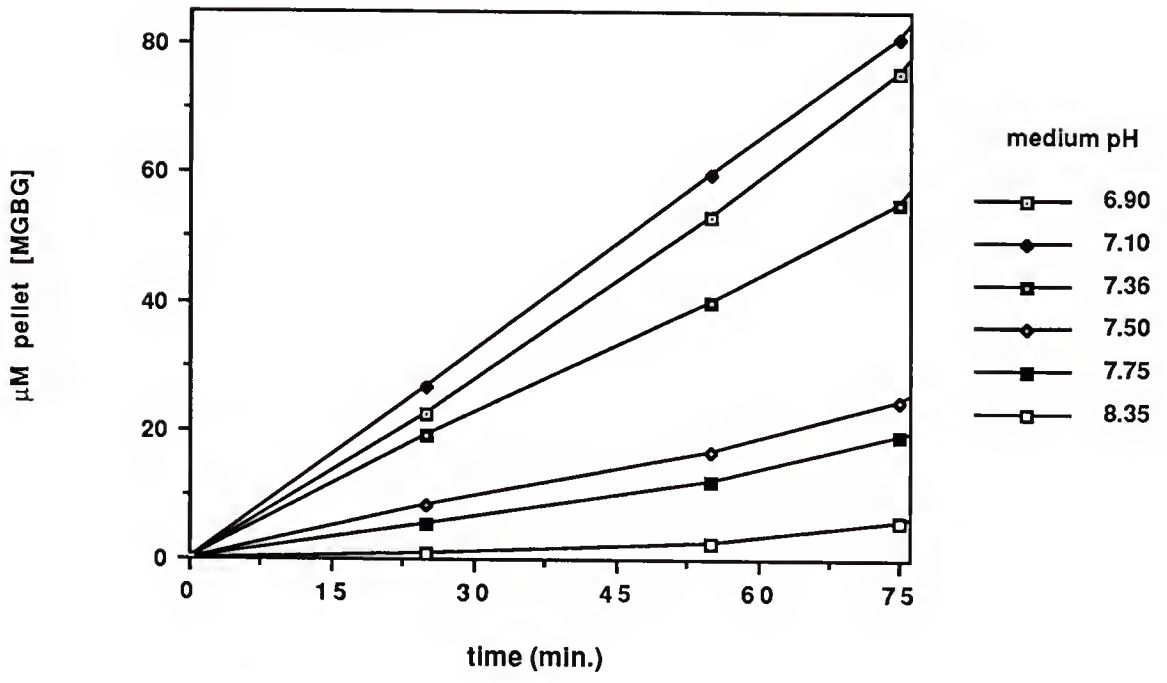

FIGURE 3-7 : Effect of extracellular pH on the initial uptake of MGBG. Logarithmically-growing L1210 cells were exposed to [^{14}C] MGBG (0.8 µM) for 0 - 75 minutes over the range of extracellular pH between 6.90 and 8.35. Intracellular radioactivity was determined at various times (0 - 75 minutes) after the exposure.

TABLE 3-2 : Comparison between the observed rates of MGBG uptake at different extracellular pH's with rates calculated on the basis of transport of $MGBG^{2+}$ only.

Extracellular pH	$[MGBG^{1+}]/[MGBG2^{+}]$ [μM]/[μM]	calculated v (μM/min.)	observed v (μM/min.)
At 0.8 μM [^{14}C] MGBG			
6.90	0.16/0.64	1.04	1.07
7.10	0.23/0.57	0.92	1.08
7.36	0.34/0.46	0.77	0.72
7.50	0.40/0.40	0.67	0.34
7.75	0.64/0.34	0.58	0.28
8.35	0.70/0.10	0.18	0.10
At 8.5 μM [^{14}C] MGBG			
6.90	1.70/6.79	5.12	5.06
7.10	2.42/6.08	4.89	5.65
7.36	3.57/4.93	4.43	4.22
7.50	4.25/4.25	4.11	2.52
7.75	5.44/3.06	3.41	1.48
8.35	7.45/1.05	1.58	0.96

Note : L1210 cells were exposed to 0.8 and 8.5 μM [^{14}C] MGBG in RPMI 1640 medium which was adjusted to different pH's with use of concentrated HCl or NaOH. Cells were incubated at 37 °C and aliquots of cell suspension were taken intermittently during the course of exposure to determine pellet radioactivity. Observed values of the uptake rate of [^{14}C] MGBG were then compared to the values calculated with an assumption that the K_m and V_{max} of MGBG uptake (4.72 μM and 8.68 μM per min., respectively) were unchanged at different pH's but apply only to $MGBG^{2+}$. The Henderson-Hassenbach equation ($pH = pK_a + \log [MGBG^{1+}]/[MGBG^{2+}]$ was used to calculate an availability of divalent form of MGBG and the Michaelis-Menten equation ($v = V_{max}$ [S]/Km + [S]), to predict the intracellular accumulation of [^{14}C] MGBG if only the divalent form of MGBG was transported into L1210 cells.

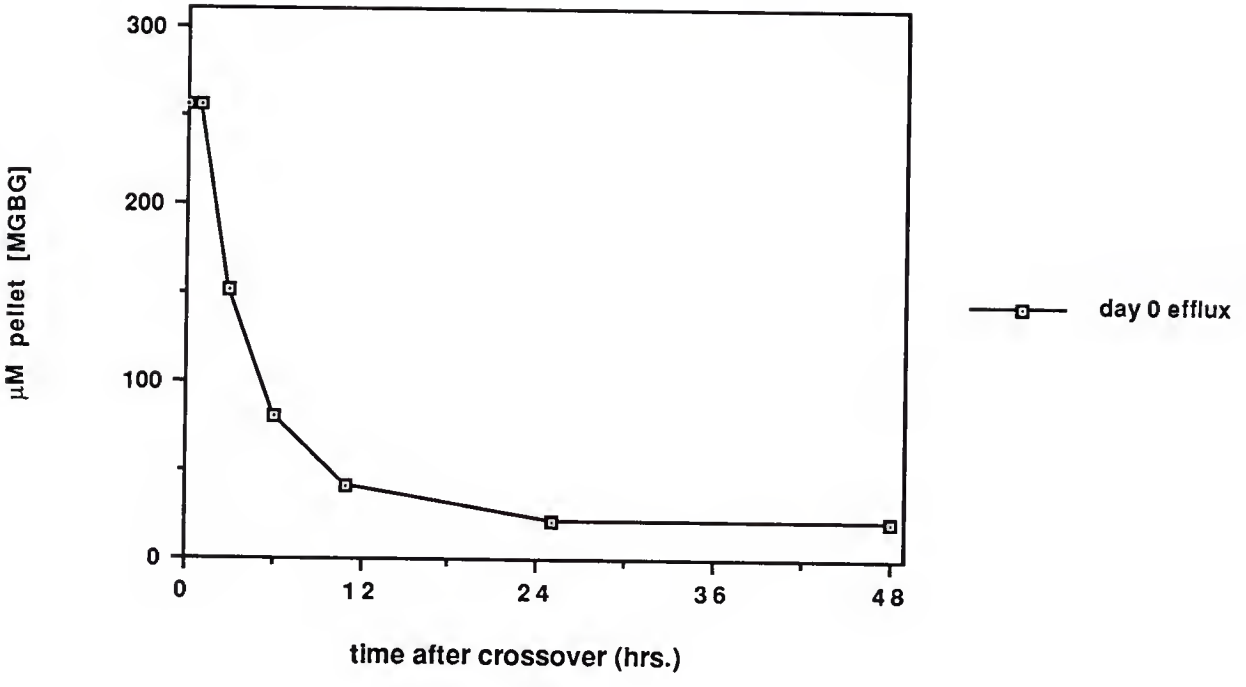

FIGURE 3-8 : Efflux of MGBG. Logarithmically-growing L1210 cells were exposed to 1.5 µM [^{14}C] MGBG for 4 hours on the first day (day 0) of incubation before centrifugation and resuspension in drug-free medium. Intracellular radioactivity was determined at various times (0 - 48 hours) after resuspension.

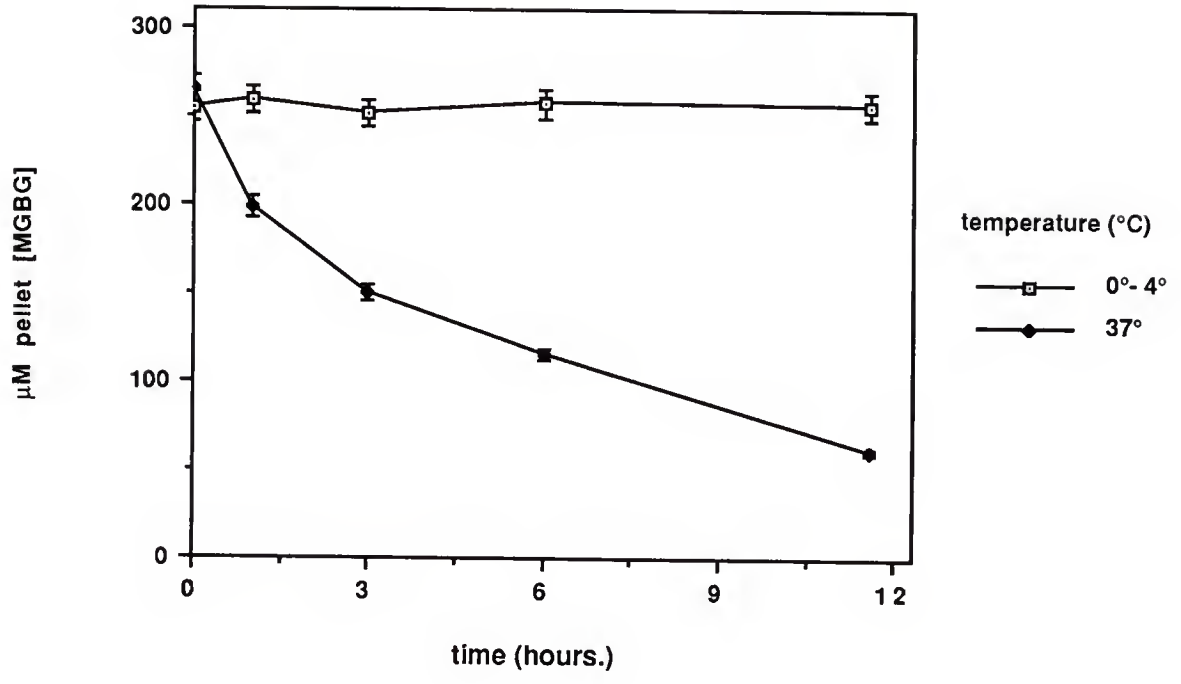

FIGURE 3-9 : Effect of temperature on the efflux of MGBG. L1210 cells at 3 x 10^5 cells per ml were incubated in medium containing 5 µM [^{14}C] MGBG for 1.5 hours at 37 °C. Cell pellet was washed once with drug-free medium at 0 - 4 °C before subsequent resuspension in drug-free medium, and the cell suspension then was incubated at 0 - 4 °C or 37 °C. Intracellular radioactivity was determined for 0 - 12 hours after the resuspension.

exposed to 5 μM MGBG for two hours before centrifugation and resuspension into medium containing 1 μM [^{14}C] MGBG. The apparent intracellular concentration of nonradioactive MGBG would be expected to be about 400 μM at the time of resuspension. The results shown in Figure 3-10 revealed that preloading the cells with MGBG did enhance modestly the uptake of [^{14}C] MGBG.

We also explored the effects of various extracellular concentrations of MGBG on the rate of efflux of [^{14}C] MGBG from preloaded L1210 cells (Figure 3-11). No stimulation of efflux was noted; when the extracellular concentration of MGBG was 1 mM, some inhibition of efflux was observed.

In summary, the presence of unlabelled MGBG inside cells stimulated the uptake of [^{14}C] MGBG, but the presence of unlabelled MGBG in the medium did not stimulate the efflux of [^{14}C] MGBG. Interpretation of this observation is quite complex because of the presence of naturally occurring polyamines in these L1210 cells.

Effects of Various Cations on the Uptake of MGBG

Sodium-dependency of MGBG uptake

The accumulation of [^{14}C] MGBG (4.0 μM) by L1210 cells was determined, at 0, 5, 15 and 30 minutes in an Earle's balanced salt solution (EBSS) which contains NaCl (116 mM). This rate of accumulation was compared to incubations in which NaCl was replaced iso-osmotically by choline choride (Figure 3-12). The results suggest that the uptake of MGBG by L1210 cells is sodium dependent. Other experiments were done to further establish this conclusion. L1210 cells were exposed to different concentrations of [^{14}C] MGBG (0.5 - 30 μM) in 10 mM HEPES-TRIS buffer, pH 7.40, which contains either 100 mM NaCl or choline chloride. The results shown in figure 3-13 revealed an MGBG concentration dependent enhancement of [^{14}C] MGBG in

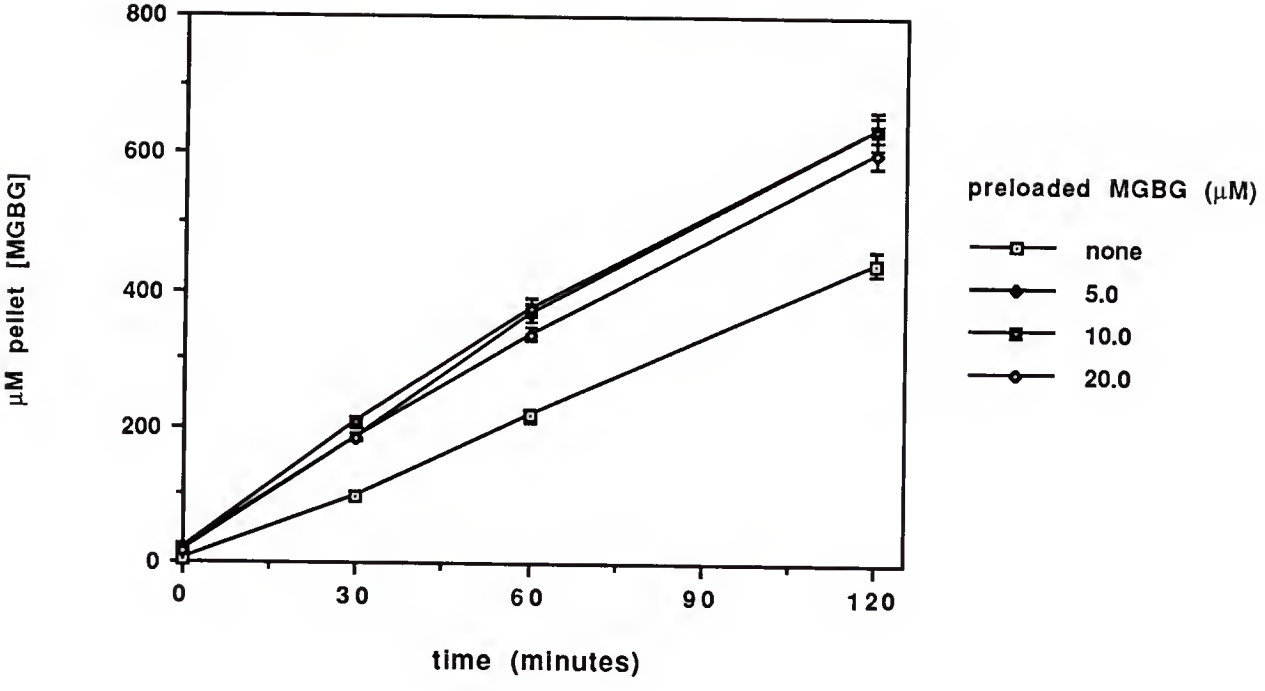

FIGURE 3-10 : Effect of MGBG preloading on the uptake of labelled MGBG. L1210 cells were exposed to 0 - 20 µM MGBG for two hours before centrifugation and resuspension into a medium containing 10 µM [^{14}C] MGBG. Intracellular radioactivity was determined for 0 - 120 minutes after resuspension.

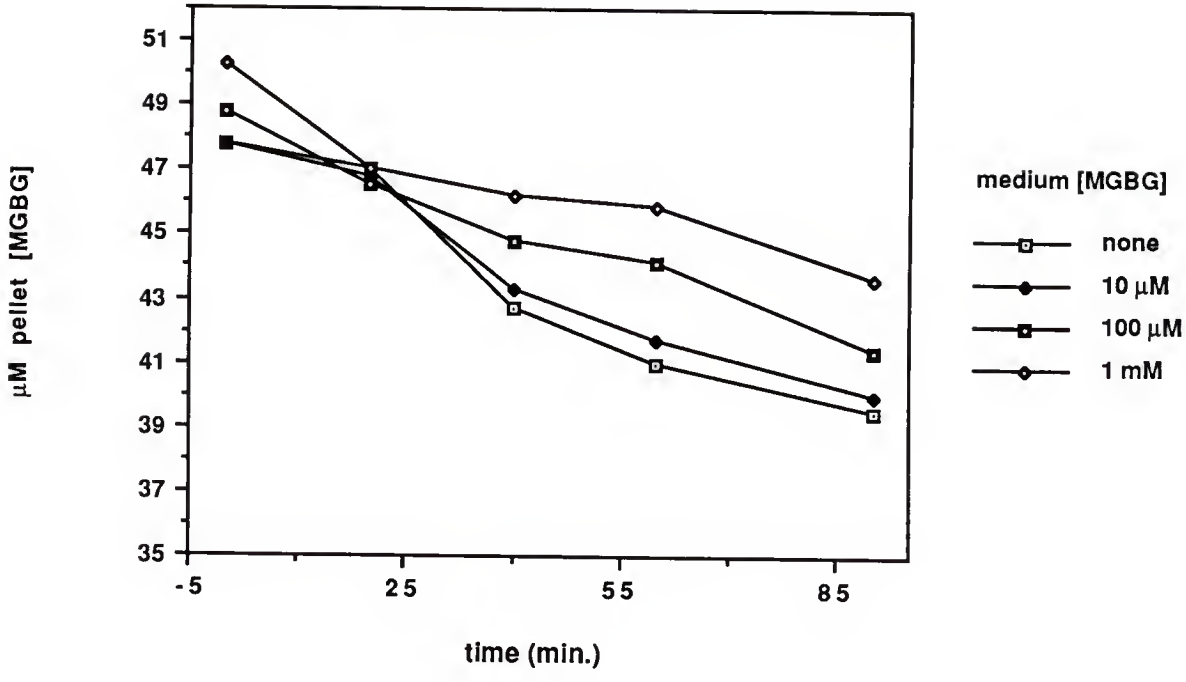

FIGURE 3-11 : Effect of medium concentration of MGBG on the efflux of labelled MGBG. L1210 cells were exposed to 1.0 µM [^{14}C] MGBG for one hour before centrifugation, and the cell pellet was resuspended into medium containing MGBG (10 µM - 1 mM). Intracellular radioactivity was determined for 0 - 90 minutes after resuspension.

the presence of NaCl. To determine kinetics of the sodium-dependent process, uptake in choline chloride was subtracted from uptake in the presence of NaCl. A Lineweaver-Burk plot of the sodium-dependent uptake as a function of [^{14}C] MGBG concentration was linear (Figure 3-14). These results indicated that the sodium-dependent component of uptake of MGBG was a single transport system with a K_m and V_{max} of 2.10 ± 0.08 μM and 226.8 ± 18.0 pmole/10^6 cells/hour, respectively. Michaelis-Menten calculations revealed that at an extracellular MGBG concentration of 1 μM, which is cytotoxic, the sodium dependent transport of MGBG accounts for more than 90 % of total uptake.

Results of experiments in which both sodium and choline are replaced by mannitol are interesting. Uptake of [^{14}C] MGBG in10 mM HEPES-TRIS buffer, pH 7.40 containing 200 mM mannitol was surprisingly high (Figure 3-15). However, Lineweaver-Burk plots of MGBG uptake as a function of MGBG concentration in the presence of mannitol, like choline chloride but unlike sodium chloride, revealed a low affinity process (high K_m).

Ability of various cations to substitute for sodium in uptake of MGBG

To determine the cation specificity of MGBG uptake, sodium chloride was replaced by a variety of monovalent cation chloride salts in incubations of L1210 cells with [^{14}C] MGBG (Figure 3-17 and Table 3-3). Choline and lithium maintained rates of uptake of MGBG about 2/3 that of sodium. Cesium and potassium were less effective in that the observed uptakes ranged from only 7 to 24 % of sodium controls. Table 3-4 depicts experiments in which MGBG uptake was assessed in the presence of varying mixtures of sodium, potassium and choline chloride. The detrimental effect of potassium, even in comparison to choline, is again apparent.

The specificity toward chloride was also examined. Substitution of sodium chloride with sodium gluconate had no effect on MGBG uptake. Replacement of

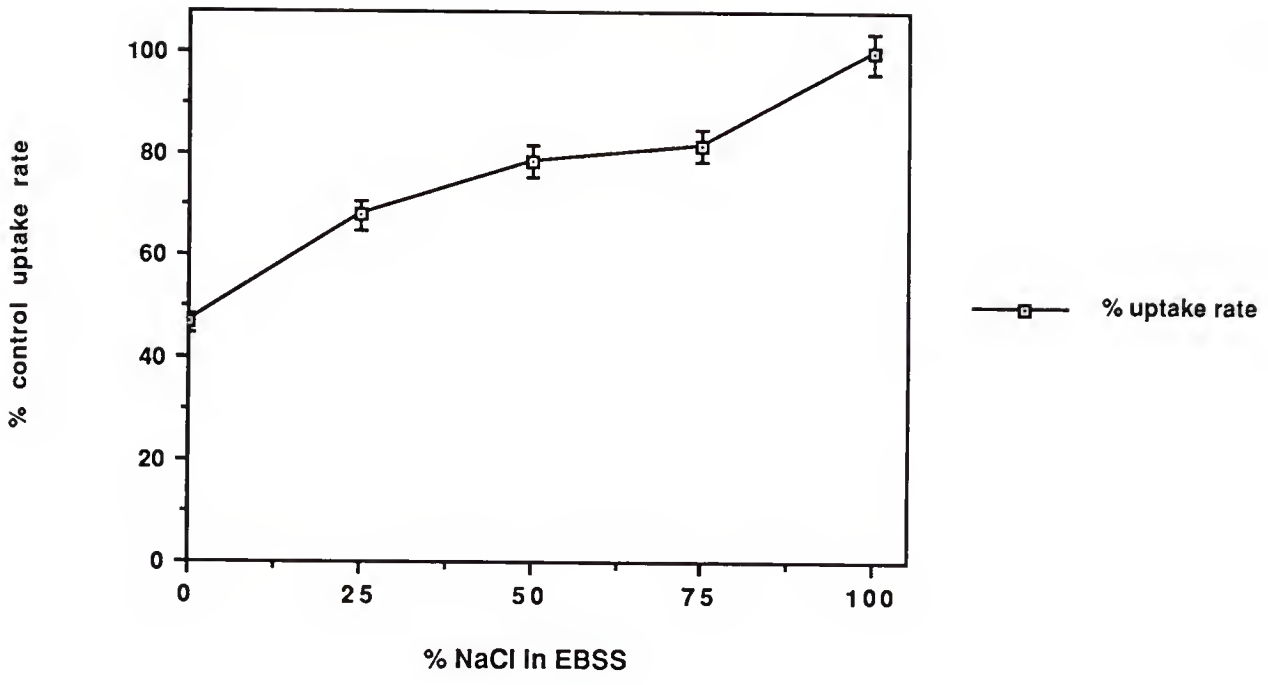

FIGURE 3-12: Sodium dependency of MGBG uptake in L1210 cells. The uptake of [^{14}C] MGBG (4.0 μM) in logarithmic-growing L1210 cells was measured in an Earle's balanced salt solution in which NaCl was iso-osmotically replaced by varying amount (0, 25, 50, 75 and 100%) of choline chloride. In each case, the uptake of MGBG was assessed at 0, 5, 15 and 30 minutes. The rate of uptake at 100% (116 mM) NaCl was set at 100% and the other rates were plotted against the fractional content of sodium. Each point represents the average of three separate experiments, with triplicate measurements in each experiment.

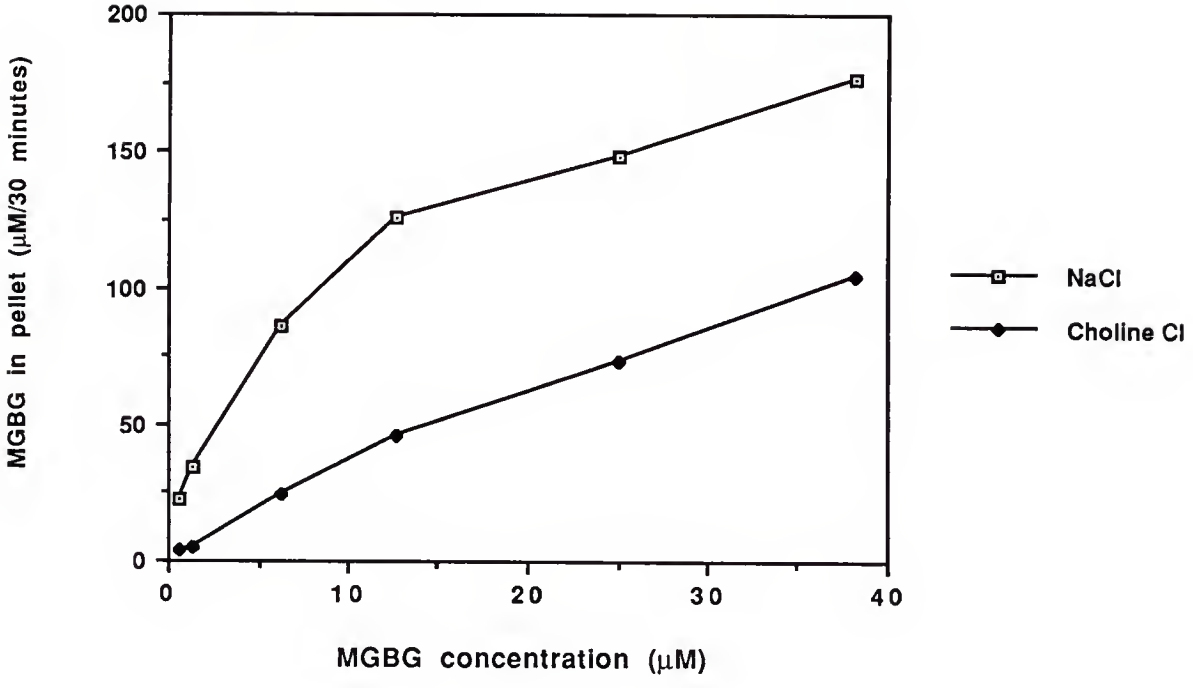

FIGURE 3-13 : Relationship between MGBG concentration and the rate of uptake of MGBG in the presence or absence of sodium. L1210 cells were exposed to different concentrations of [^{14}C] MGBG (0.5 - 30 μM) for 30 minutes in 10 mM HEPES-TRIS buffer, containing either 100 mM NaCl or 100 mM choline chloride.

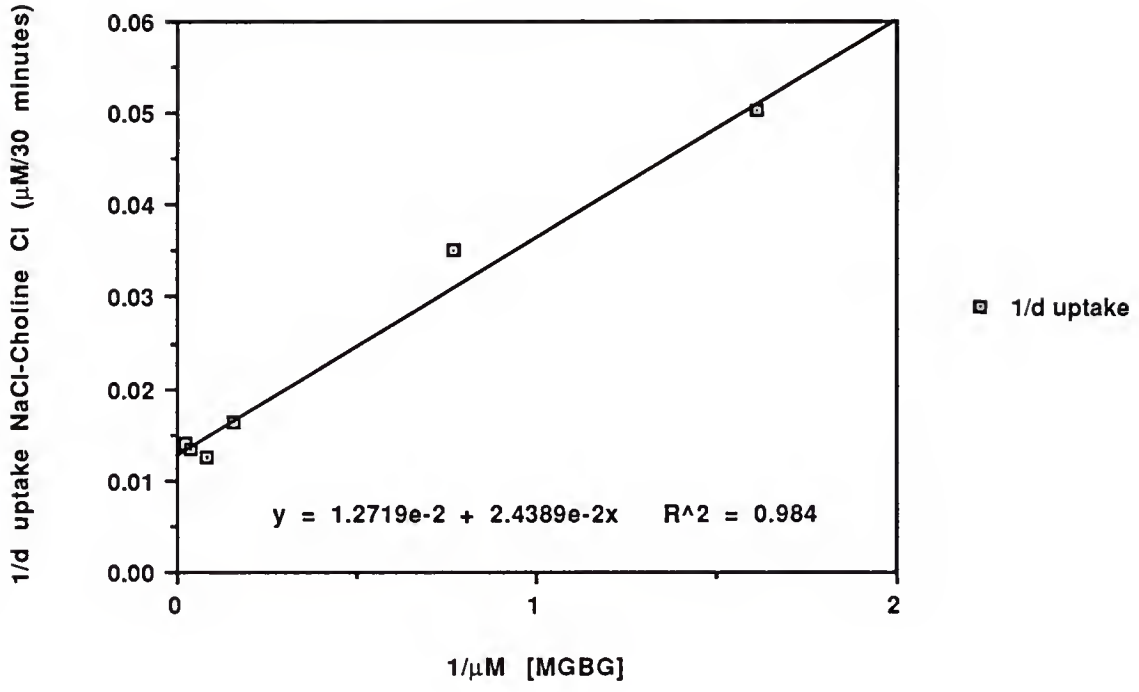

FIGURE 3-14 : Lineweaver-Burk plot of the sodium-dependent MGBG uptake in L1210 cells. Sodium-dependent uptake of MGBG refers to the diference between uptake in the presence of sodium chloride and choline chloride as indicated in Figure 3-13.

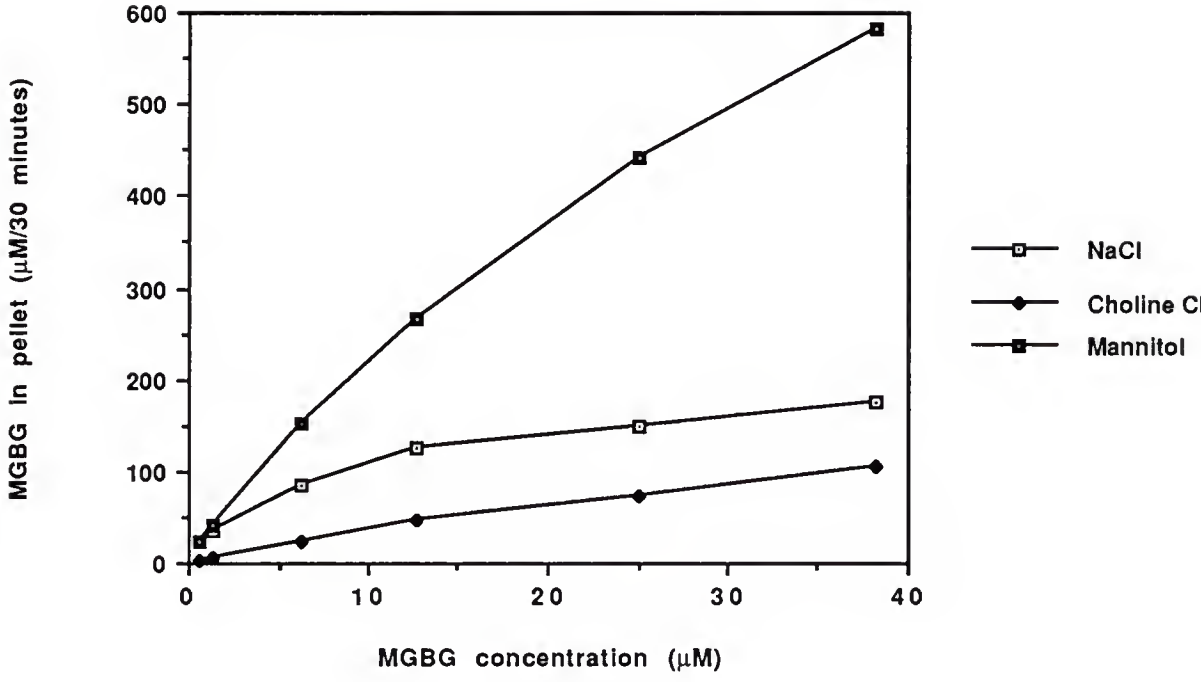

FIGURE 3-15 : The relationship between MGBG concentration and the rate of MGBG uptake in sodium chloride, choline chloride and mannitol. Cells were incubated for 30 minutes in [^{14}C] MGBG in 10 mM HEPES-TRIS buffer, pH 7.40, containing 100 mM NaCl, 100 mM choline chloride or 200 mM mannitol.

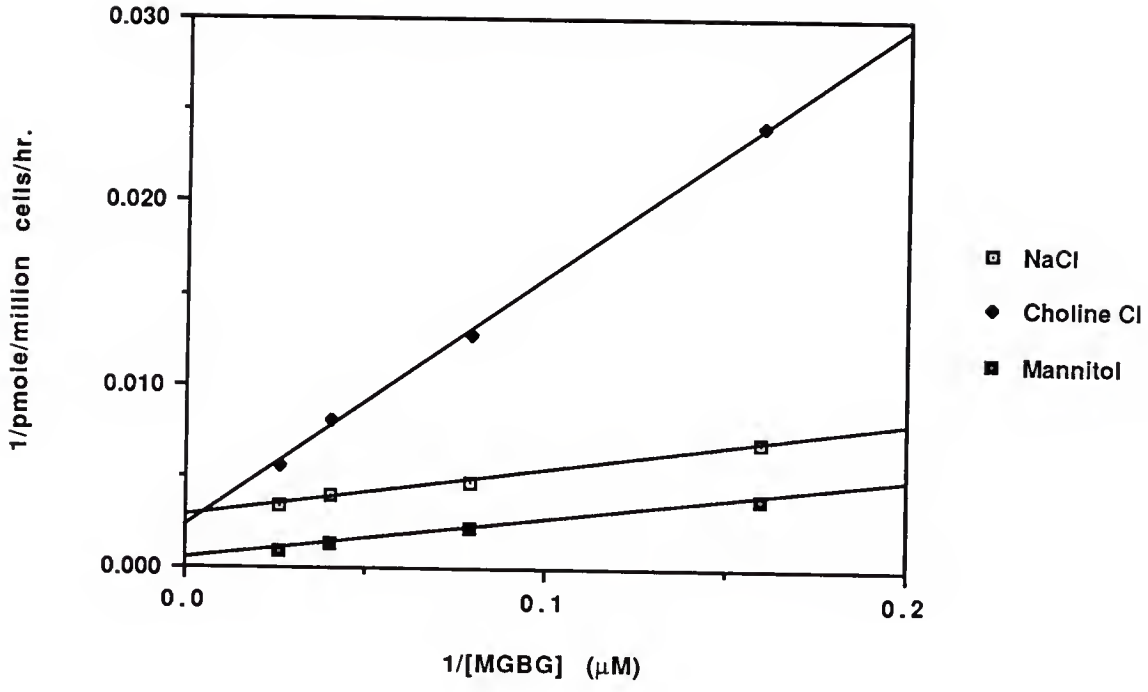

FIGURE 3-16 : Lineweaver-Burk plot of MGBG uptake in sodium chloride, choline chloride and mannitol. The data are presented in Figure 3-15. V_{max} of MGBG uptake in NaCl, choline chloride and mannitol were 362.4 $\pm$ 19.7, 450.6 $\pm$ 31.6, 2372.8 $\pm$ 103 pmole/10^6 cells/hour with K_m of 9.34 $\pm$ 1.04, 61.5 $\pm$ 2.52 and 50.7 $\pm$ 2.10 μM, respectively.

chloride ion with the relatively impermeable anion, sulfate, did not affect the initial (up to 10 minutes) uptake of MGBG, but did decrease uptake thereafter.

Effect of Ionophores on the Uptake of MGBG

Two classes of ionophores, mobile ion carriers and channel formers, with different cation specificities, were evaluated with regard to their effect on MGBG uptake. L1210 cells were preincubated with the ionophore (5 μM final concentration) for 20 minutes in Earle 's balanced salt solution before [^{14}C] MGBG was added to achieve the final concentration of 6 μM. The results are shown in table 3-5. Among the ionophores tested, gramicidin A was the most effective inhibitor of MGBG uptake. It is a channel-forming ionophore and does not discriminate between sodium and potassium ions. Therefore, it can dissipate both sodium and potassium electrochemical potential differences across the plasma membrane. In contrast, valinomycin, a neutral ionophore which is specific for the potassium ion did not significantly affect the uptake of MGBG. Effects of monensin, an ionophore specific for sodium ion, and A23187, which is specific for calcium ion (but also allows an entry of sodium and potassium ions), were intermediate. These results support the notion that a sodium electrochemical gradient across the plasma membrane is important in attaining maximal uptake of MGBG.

Effect of Inhibitors of Energy Metabolism on the Uptake of MGBG

The effect of metabolic inhibitors on the uptake of [^{14}C] MGBG was assessed in order to gain some insight into the energetics of the transport process. The inhibitors included antimycin A (an inhibitor of cytochrome reductase), 2, 4-dinitrophenol (2, 4-DNP, an uncoupler of oxidative phosphorylation), and potassium cyanide (KCN, an inhibitor of cytochrome oxidase). The effects of these inhibitors of mitochondrial energetics (0.01 mM antimycin A, 0.1 mM 2,4-DNP and 0.1 mM KCN) on drug uptake were studied by incubating L1210 cells

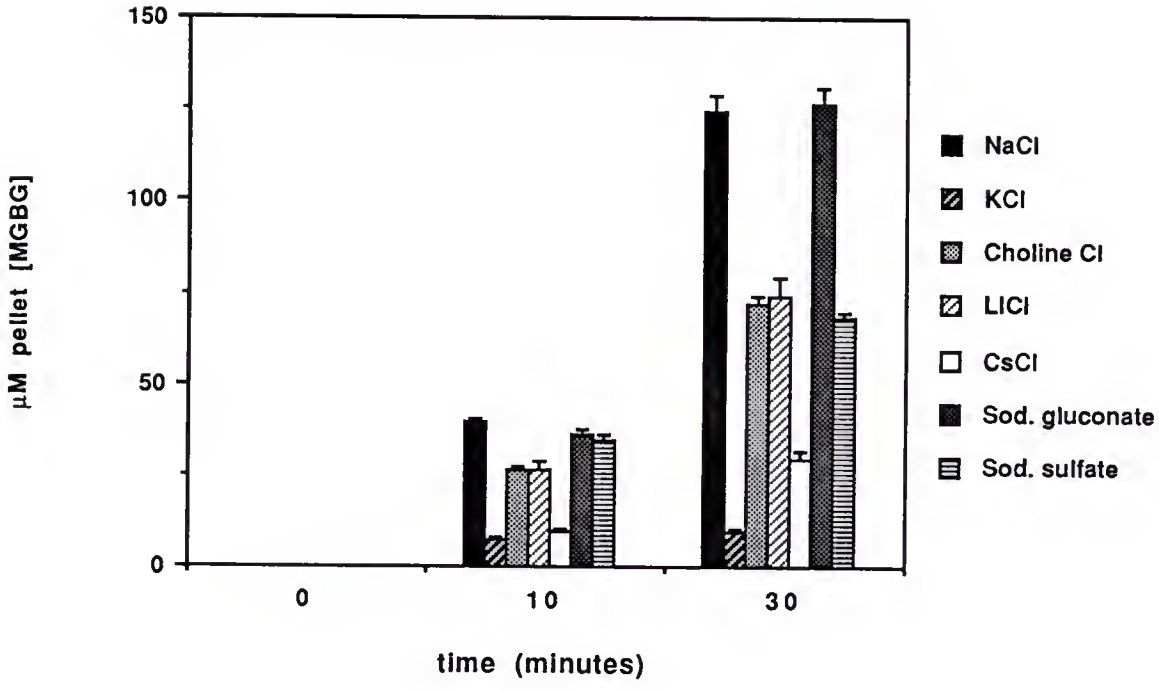

FIGURE 3-17 : Effects of various ions on MGBG uptake in L1210 cells. Incubations were performed in 10 mM HEPES-TRIS buffer, pH 7.40, containing 100 mM NaCl, KCl, choline Cl, LiCl, CsCl, Na_2SO_4 or Na gluconate. Mannitol was also added in the buffer to achieve 100 mM concentration.

TABLE 3-3 : Effects of various ions on MGBG uptake in L1210 cells.

salt	10 min. uptake		30 min. uptake	
	μM	%	μM	%
NaCl	39.2 ± 1.2	100 [a]	124.7 ± 5.2	100 [a]
KCl	7.0 ± 1.2**	17.8	9.26 ± 0.7**	7.4
Choline Cl	26.2 ± 1.5*	66.8	71.5 ± 1.3*	57.3
LiCl	26.7 ± 1.9*	68.1	74.0 ± 4.4	59.3
CsCl	9.3 ± 0.3**	23.7	29.3 ±1.2*	23.5
Na gluconate	35.8 ± 1.8	91.3	126.3 ± 3.5	101.3
Na_2SO_4	34.7 ± 0.9	88.5	67.9 ±1.61*	54.4

Note : All salts were included at a concentration of 100 mM (except Na_2SO_4, 50 μM) in the medium containing 100 mM mannitol and 10 mM HEPES-TRIS solution, pH 7.40. Results are mean ± SE of four determinations of three separate experiments. The final concentration of [^{14}C] MGBG in the incubating medium was 3.0 μM. [a] The uptake in NaCl was defined as 100 % (** $p < 0.01$, * $p < 0.05$).

TABLE 3-4 : Effects of potassium and choline chloride on the sodium-dependent MGBG uptake in L1210 cells.

EBSS treatment	percent
100 % NaCl	100[a]
50 % NaCl : 50 % choline Cl	81.0
100 % choline chloride	57.3
50 % NaCl : 50 % KCl	58.6
100 % KCl	7.4
50 % KCl : 50 % choline Cl	42.6

Note : The transport rates of [^{14}C] MGBG (3 - 4 μM) in logarithmically-growing L1210 cells were measured, respectively, for 30 - 35 minutes in an Earle 's balanced salt solution where NaCl was displaced iso-osmotically by choline chloride and/or KCl (100 % NaCl corresponds to 116 mM). At the end of incubation period, cells were washed and cell-associated radioactivity was determined as described in " Materials and General Methods ". The data represents the average of two separated experiments, with quadruplicate measurements in each experiment. [a] The uptake in 100 % NaCl was defined as 100 %.

TABLE 3-5 : Effects of ionophores on MGBG uptake in L1210 cells.

Addition	Cation Specificity	MGBG Uptake (μM/20minutes)	% Inhibition
None		24.1 ± 1.45	0
Valinomycin	K^+	23.5 ± 1.05	2.5
Monensin	Na^+	19.0 ± 0.65*	21.0
Gramicidin A	Na^+, K^+	10.0 ± 0.45**	58.3
A23187	Ca^{++}	16.4 ± 0.4*	32.1

Note : L1210 cells were preincubated with the individual ionophores (5 μM) for 20 minutes in Earle's balanced salt solution prior to an addition of [^{14}C] MGBG (6 μM final concentration). MGBG uptake was measured at 0, 20 and 40 minutes after adding MGBG. Pellet radioactivity was determined as described earlier. Each value represents the mean ± SEM, of three separate experiments, each conducted in triplicate (* $p < 0.05$, ** $p < 0.01$).

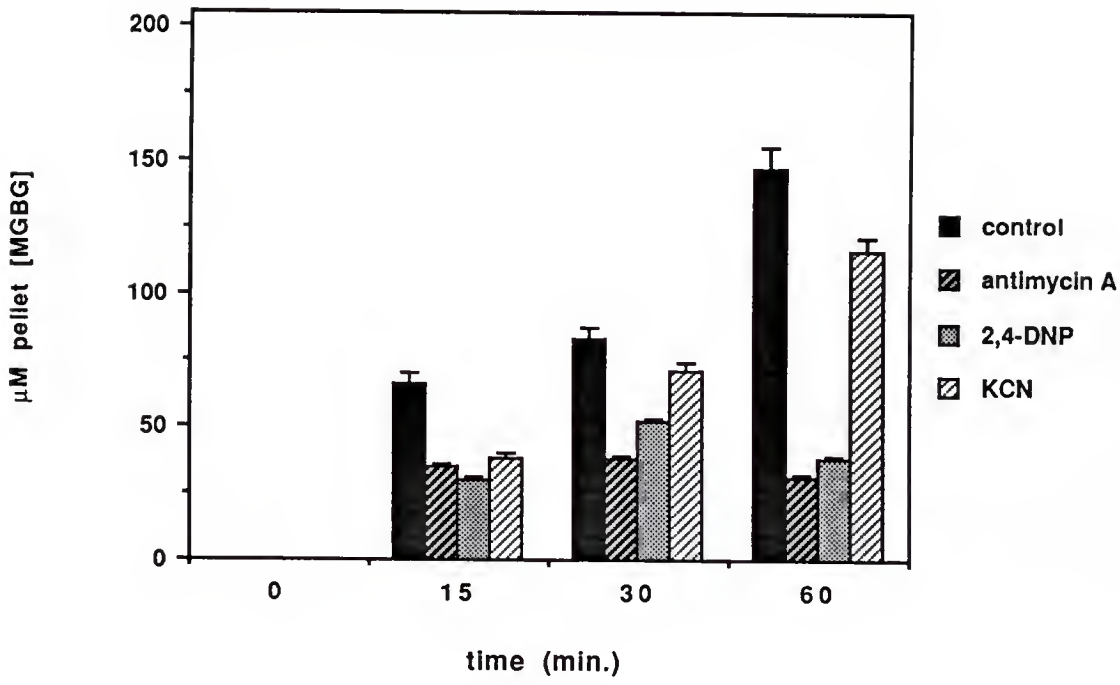

FIGURE 3-18 : Effects of inhibitors of energy metabolism on MGBG uptake. L1210 cells were pretreated with 0.01 mM Antimycin A, 0.1 mM 2,4-DNP or 0.1 mM KCN in PBS solution for 20 minutes, prior to addition of [^{14}C] MGBG (5 µM final concentration). The cells were incubated in [^{14}C] MGBG for the indicated times. Values are expressed as intracellular concentrations of MGBG (µM).

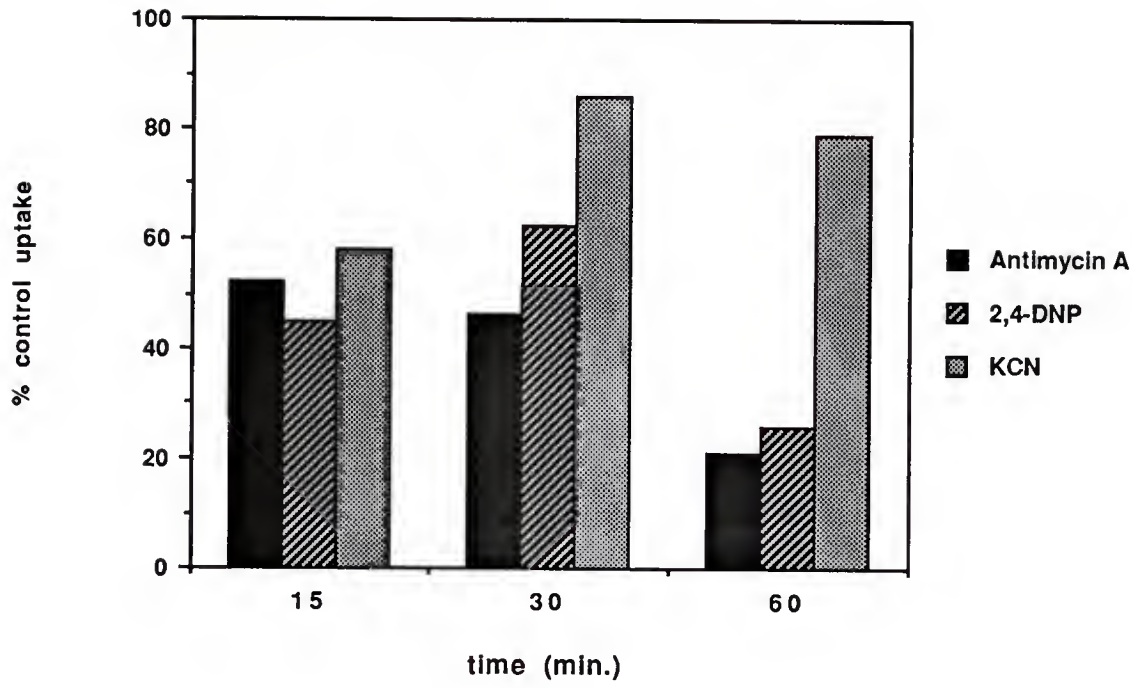

FIGURE 3-19 : Effects of inhibitors of energy metabolism on MGBG uptake. L1210 cells were pretreated with 0.01 mM Antimycin A, 0.1 mM 2,4-DNP or 0.1 mM KCN in PBS solution for 20 minutes, prior to addition of [^{14}C] MGBG (5 μM final concentration). The cells were incubated in [^{14}C] MGBG for the indicated times. Values are expressed as percent of control (no inhibitor).

with the inhibitors for 20 minutes at 37°C prior to the addition of [^{14}C] MGBG. These uptake studies were performed in the phosphate-buffered saline without glucose to minimizing energy derived from glycolysis. The uptake of drug at 15, 30 and 60 minutes is expressed as an intracellular concentration of MGBG (μM) in Figure 3-18 and as a percentage of control uptake in Figure 3-19. All are corrected for rapid binding. The results reveal a significant decrease in the accumulation of [^{14}C] MGBG in the cells pretreated with the metabolic inhibitors (antimycin >> 2,4-DNP > KCN). The data suggest a requirement of energy for the drug uptake.

Intracellular Distribution of MGBG

I was particularly interested in exploring whether or not the striking intracellular accumulation of MGBG reflected accumulation of the drug into mitochondria. Two factors prompted this interest: 1) the cationic nature of MGBG; and 2) the effects of MGBG on mitochondrial structure, function and replication. Attempts to preload the cells with [^{14}C] MGBG and then isolate mitochondria were not pursued vigorously because the procedure involved in the preparation of a mitochondrial fraction from these cells would predispose to redistribution of radiolabel. Rather, two other experimental approaches were taken: 1) uptake of [^{14}C] MGBG by isolated L1210 mitochondria; and 2) digitonin permeabilization of the plasma membrane of cells preloaded with [^{14}C] MGBG.

Uptake of MGBG by Isolated Mitochondria

Exponentially-growing L1210 cells were incubated with 0.1% w/v digitonin in dimethylsulfoxide (DMSO) until approximately 70% of the cells no longer excluded trypan blue. Cells were then washed once with a solution containing 250 mM sucrose, 2 mM EDTA and bovine serum albumin (1 mg/ml) at pH 7.40. The cell pellet was immediately homogenized on ice with a 2 ml tapered glass tissue grinder until more than 90% of the cells were ruptured. The homogenate

was centrifuged at 1000g for 10 minutes. The resulting supernatant fraction was centrifuged at 16,000g for 15 minutes to obtain the mitochondrial fraction.

The respiratory control ratio (RCR) or the state 3/state 4 respiratory ratio of the mitochondrial fraction was 2.0 to 2.5; with intact cells the RCR was 3.0 to 3.5. Oxygen consumption was measured with an oxygen electrode with succinate as substrate in an incubation medium containing 150 mM sucrose, 25 mM glycylglycine, 40 mM KCl, and bovine serum albumin (1 mg/ml).

Mitochrondrial pellets were resuspended in 2.5 μM [^{14}C] MGBG in RPMI 1640 medium at 37°C, pH 7.40. Radioactivity in the mitochondrial pellet was measured after 0, 0.5, 1, 2, and 3 hours of incubation. The mitochrondrial pellet was collected by centrifugation and washed once in medium containing 1 mM unlabelled MGBG before recentrifugation. The uptake of [^{14}C] MGBG by mitochondria (in pmoles per mg protein) is presented in Table 3-4. The concentration of [^{14}C] MGBG in the mitochondrial pellet even after three hours of exposure to 2.5 μM drug is less than 2.5 μM (for cells, about 15 pmoles per mg protein is equivalent to 2.5 μM MGBG). Furthermore, mitochondrial uptake is not likely to be the driving force for sequestration of MGBG within the cell because the observed rate of uptake is only about 1% of that predicted by the kinetic constants determined by experiments with intact cells.

Selective Release of Cytosolic Constituents by Digitonin

Because of its low content of cholesterol, the inner membrane of mitochondria is more resistant than the plasma membrane to disruption by digitonin. I used this difference to assess whether or not the MGBG that had accumulated within L1210 cells was predominately localized in mitochondria; rhodamine 123 was used as a positive control since it is well established that this cationic dye is highly concentrated within mitochondria (Johnson et al., 1981).

L1210 cells were exposed to 0.5 μM [^{14}C] MGBG for 2 to 8 days. In addition to determination of the apparent intracellular concentration of MGBG, one-ml aliquots of cells were admixed gently in an ice bath with enough 0.1% (w/v) digitonin in DMSO to cause 90 to 95% of the cells to become permeable to trypan blue. The [^{14}C] MGBG remaining in the pellet of the intact and permeabilized cells was compared (Figure 3-20). Less than 6% of the drug in the intact cells was recovered in the pellet of cells with disrupted plasma membranes.

Concurrent experiments with rhodamine 123 provided evidence that a compound that is accumulated by mitochondria remained within the cell after this treatment with digitonin. One million cells were pelleted and resuspended in 100 μl of serum-free medium. Rhodamine 123 (2-[6-amino-3-imino-3H-xanthen-9-yl]-benzoic acid methyl ester) (Sigma Chemical) was added at the final concentration of 1 μg/ml. The samples were incubated at 37°C for 10 minutes and washed once by centrifugation. The stained cells were observed under a Zeiss epifluorescence microscope and were virtually indistinguishable (in terms of mitochondrial fluorescence at an excitation wavelength of 485 nm) from cells not disrupted by digitonin.

The experiments with both isolated mitochondria and digitonin lead me to conclude that the large accumulation of MGBG within the cells involved active transport of MGBG across the plasma membrane, some degree of intracellular binding, and passive equilibration with the various intramitochondrial compartments.

Effect of Growth Rate of L1210 cells on MGBG Transport

L1210 cells were seeded at an initial concentration of 3 x 10^4 cells/ml and incubated at 37°C for five days without reseeding or adding fresh medium. The cells divided exponentially until their concentration reached 1.8 x 10^6 cells/ml

TABLE 3-6 : Uptake of MGBG by isolated mitochondria [a].

Time of incubation in MGBG (hrs)	Mitochondrial MGBG (pmole/mg protein)	% of expected intracellular MGBG [b]
0.5	0.50 ± 0.02*	0.36
1	3.83 ± 0.04	1.35
2	1.94 ± 0.07	0.35
3	2.68 ± 0.06	0.32

Note : [a] The mitochondrial fraction obtained from L1210 cells was incubated in 2.5 μM [^{14}C] MGBG for 0 - 3 hrs. The radioactivity in the mitochondrial pellet was determined after two washes with ice-cold 1 mM MGBG. The results were expressed as pmole mitochondrial [^{14}C] MGBG/mg protein (* mean ± SEM).

[b] I assumed for this calculation that the uptake of MGBG by L1210 cells was due totally to accumulation of the drug in mitochondria. The kinetic constants obtained from experiments with intact cells were used to predict rates of uptake by mitochondria. It can be seen that the actual rates of uptake were only about 1 % of the prediction.

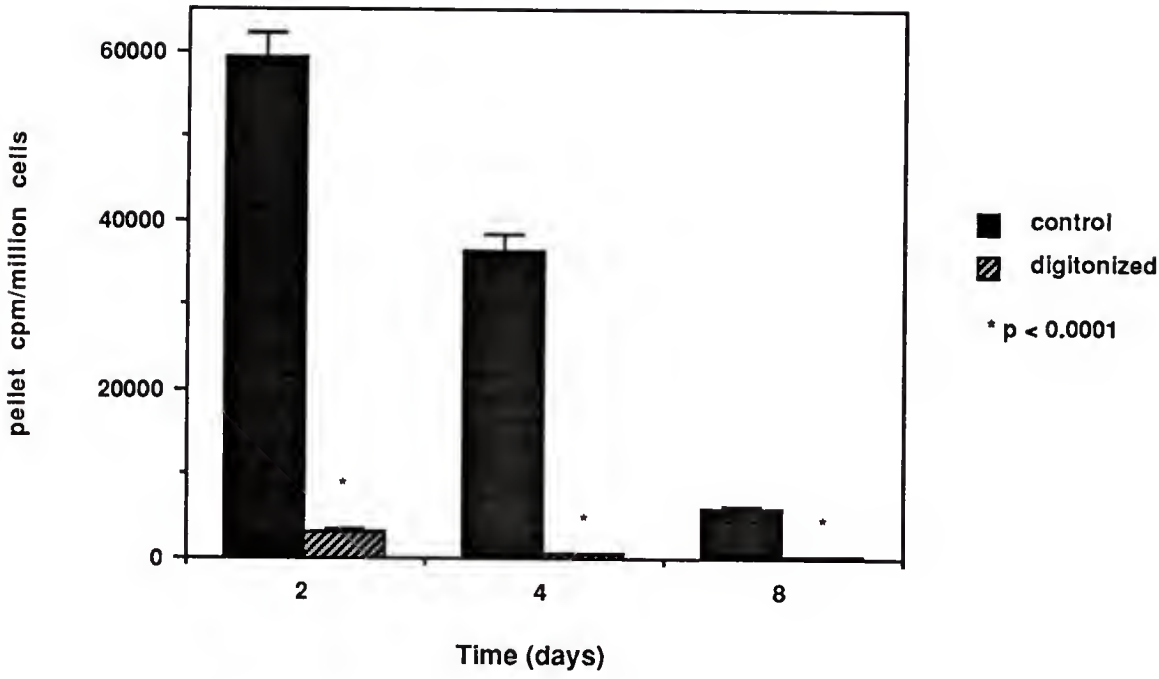

FIGURE 3-20 : MGBG remaining in the cell pellet after permeabilization of the plasma membrane by digitonin. L1210 cells were exposed to 0.5 μM [^{14}C] MGBG for 2 to 8 days. Prior to determination of the apparent intracellular concentration of MGBG, one-ml aliquots of cells were admixed gently in an ice bath with enough 0.1% (w/v) digitonin in dimethylsulfoxide until approximately 95% of the cells became permeable to trypan blue dye. The [^{14}C] MGBG remaining in the pellet of the intact and permeabilized cells was determined and compared.

(Figure 3-21). Plateau growth ensued when the concentration of cells reached 2 x10^6 cells/ml. Cell viability exceeded 95% when the cells were in logarithmic growth, but it declined progressively to 40% by the fifth day of incubation when the cells were at plateau (Figure 3-22).

Cells in logarithmic growth were compared to those in early plateau with regard to transport of MGBG. L1210 cells at the two growth rates were reseeded in fresh media containing 0.5 µM [^{14}C] MGBG at starting concentrations of 1 x 10^6 and 2 x 10^6 cells/ml, respectively. The intracellular content of radiolabel was determined at 0, 24 and 48 hours after the start of incubation at 37°C, pH 7.40. The accumulation of MGBG was higher in the logarithmically-growing cells (Figure 3-22). Kinetic experiments with cells just entering plateau phase revealed that the decrease in uptake of MGBG in these cells reflected more on an increase in K_m (9.04 ± 0.34 µM, $P < 0.01$) than a decrease in V_{max} (0.271 ± 0.019 nmoles/10^6 cells/hour) relative to cells in exponential growth.

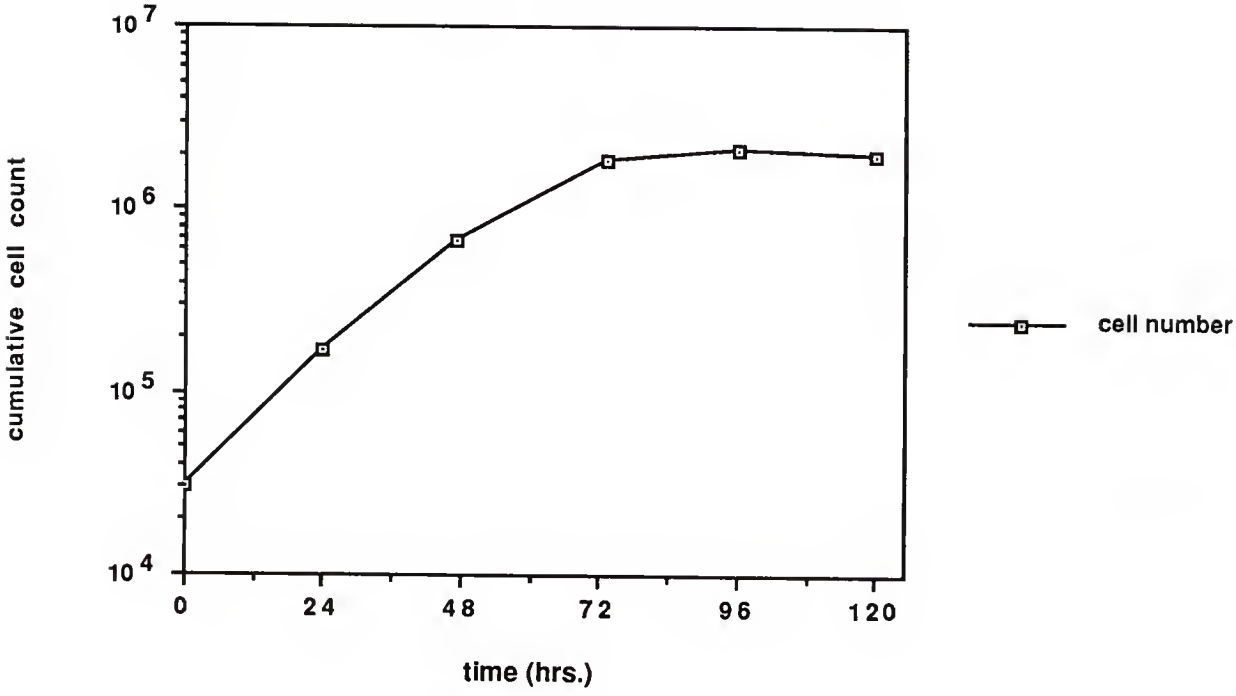

FIGURE 3-21 : Growth curve of L1210 cells. L1210 cells were seeded at an initial concentration of 3 x 10^4 cells/ml and incubated for 5 days without reseeding or adding fresh medium. Cell counts were determined by electronic particle analysis (Coulter Counter).

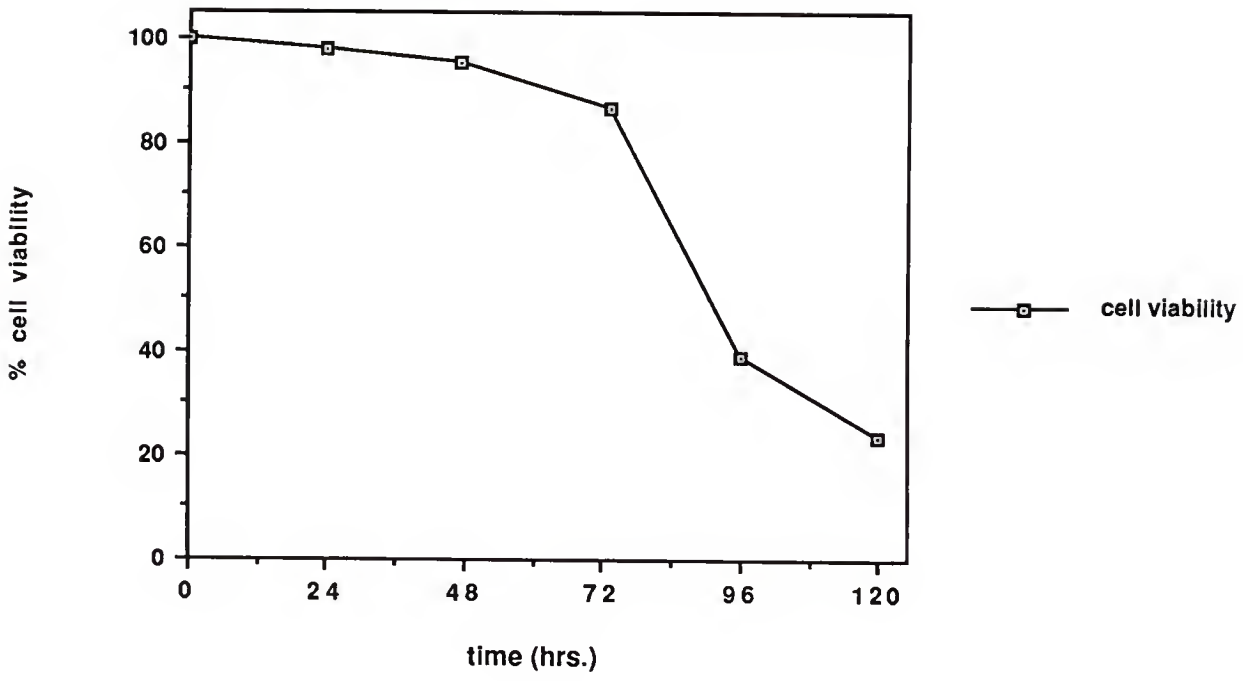

FIGURE 3-22 : Viability of L1210 cells as a function of growth phase. L1210 cells were seeded at an initial concentration of 3 x 10^4 cells/ml and incubated for 5 days without reseeding or adding fresh medium. Cell viability was determined with use of Trypan Blue dye exclusion method.

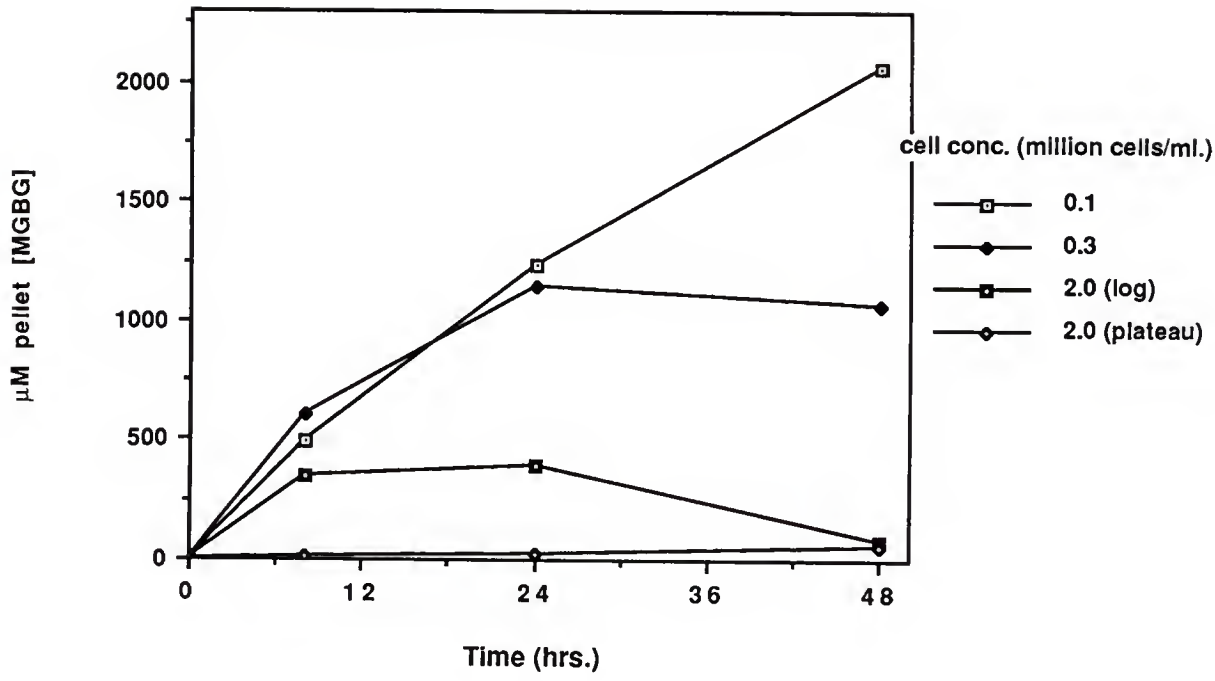

FIGURE 3-23 : Uptake of MGBG as a function of growth rate. Exponentially growing L1210 cells were seeded at initial concentrations of 0.1 - 2.0 x 10^6 cells/ml to attain different subsequent rate of growth. In addition, cells already in the plateau phase were seeded at 2.0 x 10^6 cells/ml. All cells were exposed to fresh medium containing 0.5 µM [^{14}C] MGBG at starting concentrations of 1 x 10^5, 3 x 10^5 and 2 x 10^6 cells per ml. The intracellular content of radiolabel was determined at 0, 24, and 48 hours after the start of incubation.

CHAPTER 4

TRANSPORT OF MGBG AND DRUG RESISTANCE

When L1210 cells were treated with MGBG at concentrations that were low enough (< 5 μM) to allow survival of some cells, the residual cells reacquired the control rate of proliferation within one to three weeks (Figures 3-1 and 3-2) despite the continued presence of MGBG in the medium. As will be documented below, this resistance seems to reflect a decreased intracellular/extracellular concentration ratio of MGBG. This Chapter describes the changes that account for this decrease in the intracellular accumulation of MGBG. As discussed in Chapter 3, MGBG had accumulated in cells for the first 20 hours of exposure to drug in accord with a saturable process with a V_{max} of 296 ± 19 pmole/10^6 cells/hour and a K_m of 4.72 ± 0.17 μM. Our focus now turns to subsequent 19 days of exposure.

Concentration of MGBG in L1210 Cells During 20 Days of Drug Exposure

This experiment resembles that depicted in Figure 3-1 in which the effects of varying concentrations of MGBG on L1210 cell growth were ascertained except that the current experiment incorporated the use of [^{14}C] MGBG to allow intermittent measurement of radiolabel in the cell pellet. Logarithmically-growing L1210 cells were reseeded every two days at a density of 8 x 10^4 cells/ml in media at 37°C, pH 7.40 containing 0.1 to 5.0 μM [^{14}C] MGBG. MGBG, 0.1 μM, has minimal, if any, effect on growth or viability. MGBG, 5.0 μM, is occasionally compatible with long-term cell survival. The intracellular content

of [^{14}C] MGBG was determined intermittently (Figure 4-1). The apparent intracellular concentration of MGBG depended both on the concentration of drug added to the medium and on the duration of exposure. At 1.0 μM MGBG in the medium, the intracellular concentration of drug reached 2000 μM about 24 hours after the start of incubation (as described in Chapter 3). Despite continued presence of the drug in the medium (including reseeding the cells into fresh drug every 48 hours), the intracellular concentration of MGBG decreased to 500 μM by day 6 and 100 μM by day 20. The same pattern applied to all doses of MGBG as long as they were high enough to affect cell growth; peak intracellular concentrations occurred between 24 and 72 hours after the start of the incubation and decreased thereafter. When the concentration of MGBG was too low to have a detectable effect on the growth rate or viability of L1210 cells, no such maximum was seen.

Although it is tempting to attribute, or at least relate, this decrease in intracellular content of MGBG to the decrease in growth rate that occurs a few days after the start of exposure to MGBG, the rate of cell proliferation returned to the control value within two weeks of exposure to MGBG (Figure 3-1), a time when the intracellular/extracellular ratio of MGBG is still decreasing.

Intracellular content of putrescine, spermidine and spermine in L1210 cells was determined with use of high performance liquid chromatography (HPLC) in our laboratory by M. Kelly (Table 4-1). By 48 hours of exposure in 0.5 μM MGBG, the intracellular content of putrescine increased while the levels of spermidine and spermine had decreased. This result was anticipated because MGBG is an effective inhibitor of S-adenosylmethionine decarboxylase. By day 20, when rate of cell proliferation had recovered, the intracellular concentrations of polyamines had also returned to control.

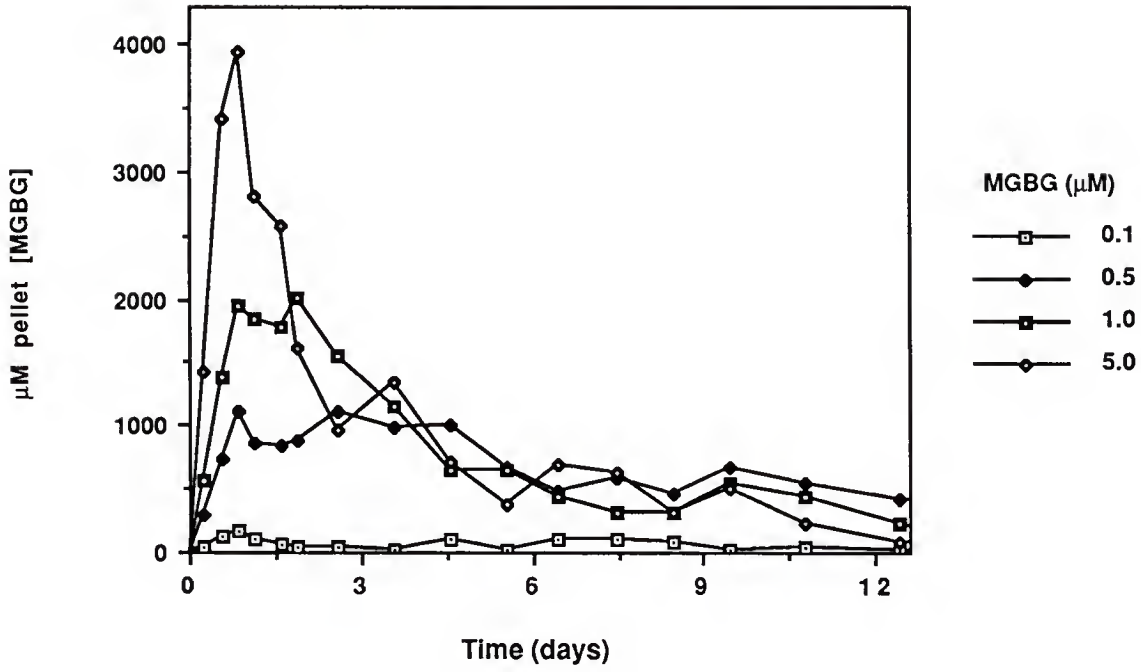

FIGURE 4-1 : Intracellular content of MGBG upon prolonged exposure to the drug. Logarithmically-growing L1210 cells were reseeded every two days at a density of 8 x 10^4 cells/ml in media containing 0.1 to 5.0 µM [^{14}C] MGBG. The intracellular content of [^{14}C] MGBG was determined periodically.

TABLE 4-1 : Effects of MGBG on the intracellular content of polyamines and putrescine in L1210 cells.

Treatment	Intracellular content (pmole/million cells, mean ± SEM)			
	Putrescine	Spermidine	Spermine	Total
Control	282 ± 6	2577 ± 81	612 ± 17	3483 ± 120
Cells treated with 0.5 μM MGBG for 48 hours				
	1838 ± 49**	1334 ± 39*	344 ± 35*	3574 ± 82
Cells treated with 0.5 μM MGBG for 20 days (after recovery)				
	244 ± 27	2494 ± 201	537 ± 26	3275 ± 249

Note : Intracellular polyamine and putrescine content was determined with use of high performance liquid chromatography ; ** $p < 0.01$, * $p < 0.05$ when compared to the control.

Kinetic Characterization of MGBG Transport as Resistance Develops

An experiment was designed to compare rates of influx and efflux of MGBG before and after the peak intracellular concentration had been attained. The cells were studied 4 hours and 156 hours (6.5 days) after the start of incubation in 1.5 μM MGBG. The experiment was designed in this way in order to compare transport in cells with an intracellular concentration of MGBG of about 400 μM on the increase (4 hours) and on the decrease (156 hours). To be able to study both influx and efflux in the same sets of cells, the L1210 cells were incubated in either radioactive or nonradioactive MGBG and crossed over at 4 hours or 156 hours. For example, to study efflux after exposure to MGBG for 156 hours, cells were incubated in 1.5 μM [^{14}C] MGBG for 156 hours at which time the intracellular concentration of [^{14}C] MGBG is about 400 μM. The cells were then collected, washed quicky, and reseeded in fresh medium containing 1.5 μM nonradioactive MGBG , and release of label into the medium was followed.

The results depicted in Figures 4-2 and 4-3 reveal that the rate of influx of MGBG had decreased more than two fold between 4 and 156 hours whereas the rate of efflux remained unchanged. Within 156 hours of MGBG exposure, the rate of influx was approximately the same as the rate of efflux (Figure 4-4), i.e. steady-state had nearly been obtained. This observation explains the relative stability of intracellular concentrations of MGBG thereafter.

A more detailed kinetic analysis was obtained with cells that had been exposed to 0.5 μM MGBG for one month. After one month of such treatment, the K_m for MGBG uptake at 37°C, pH 7.40 had increased from 4.7 ± 0.2 to 24.9 ± 1.4 μM ($P < 0.01$) and the V_{max} had decreased from 296 ± 19 to 142 ± 13 pmole/10^6 cells/hour ($P < 0.01$). If the cells were incubated in 1.0 μM MGBG, the calculated rate of influx of drug would be less than 10% of what it had been during the first 20 hours of exposure. These changes in the kinetic properties of

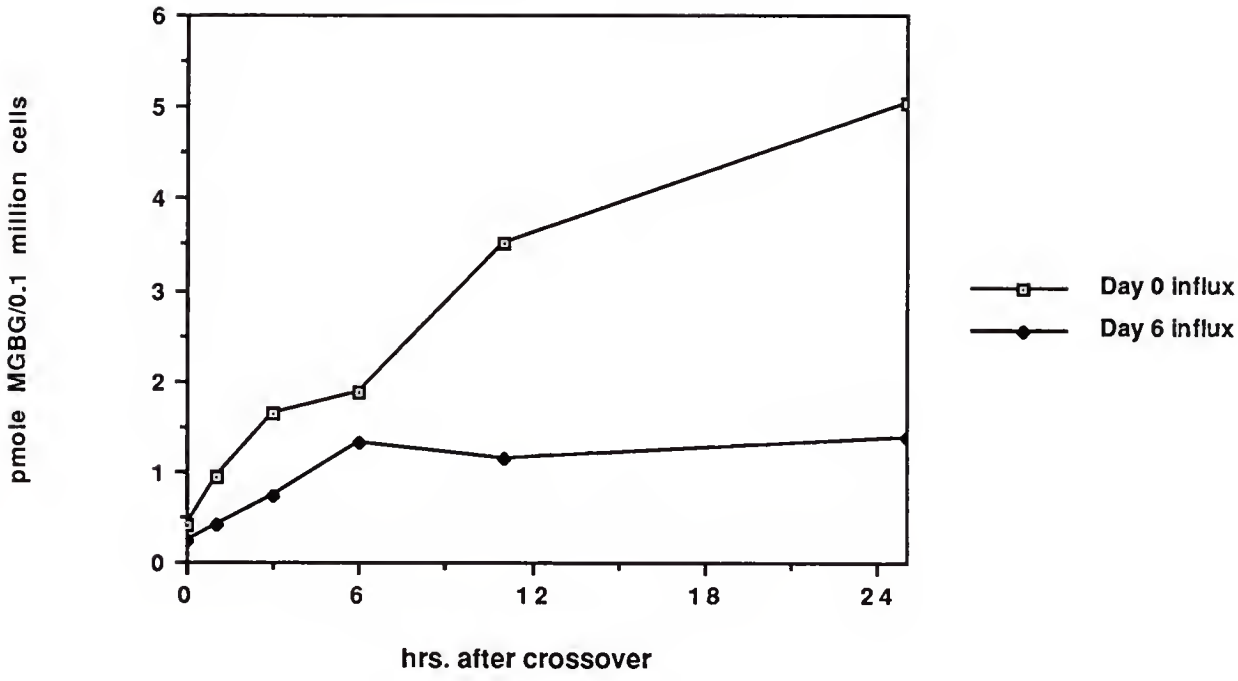

FIGURE 4-2 : Influx of MGBG before and after the peak intracellular concentration of drug had been attained. L1210 cells were incubated in 1.5 μM MGBG for 4 or 156 hours before centrifugation, washing, and resuspension in fresh medium containing 1.5 μM [^{14}C] MGBG. The intracellular content of [^{14}C] MGBG was determined periodically from 0 - 26 hours after crossover.

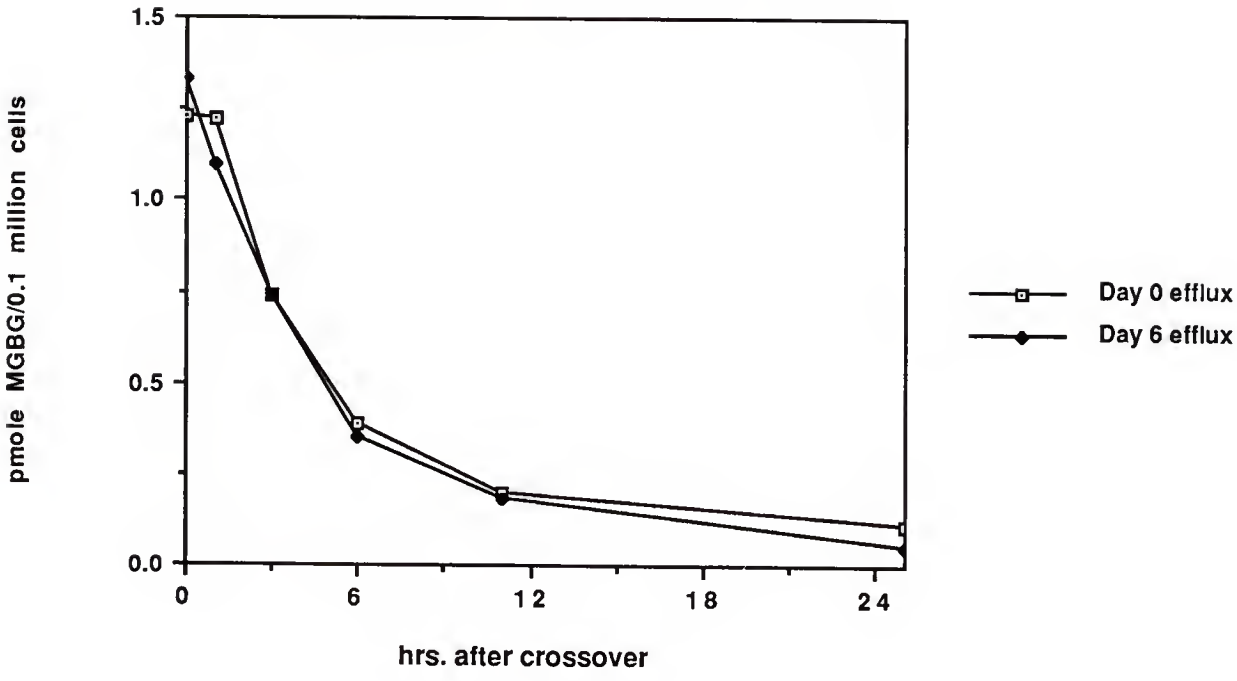

FIGURE 4-3 : Efflux of MGBG before and after the peak intracellular concentration of drug had been attained. L1210 cells were incubated in 1.5 μM [^{14}C] MGBG for 4 or 156 hours before centrifugation, washing, and resuspension in fresh medium containing 1.5 μM MGBG. The intracellular content of [^{14}C] MGBG was determined intermittently for 0 - 26 hours after crossover.

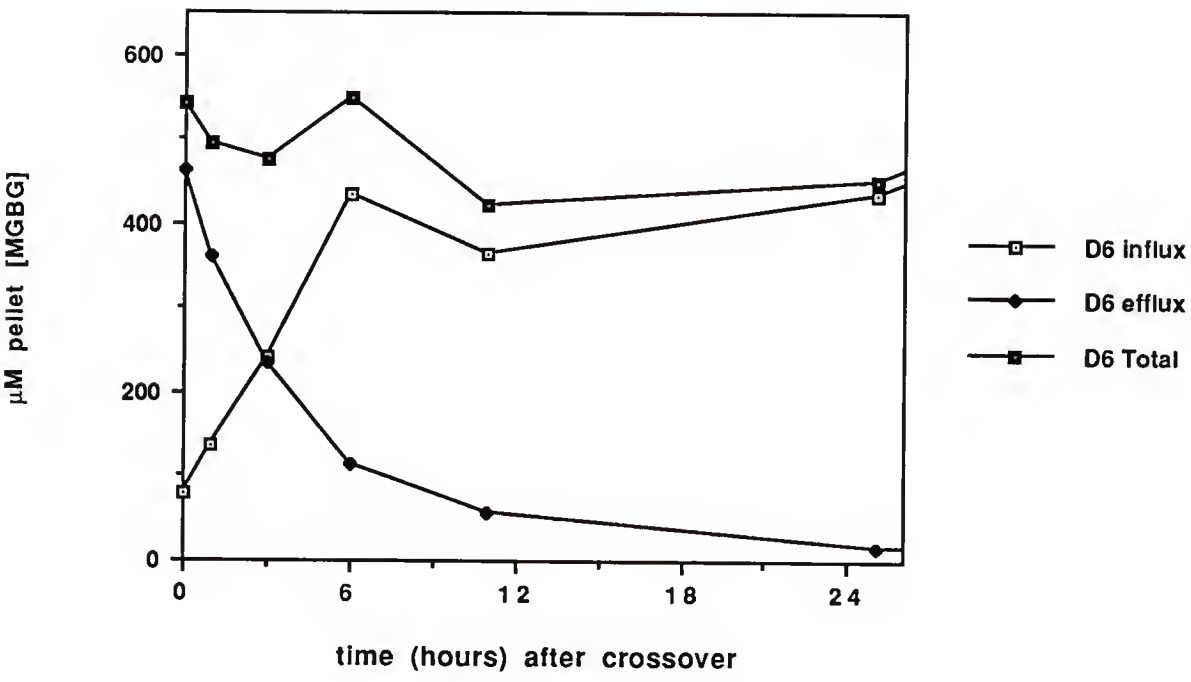

Figure 4-4 : Influx, efflux and apparent steady-state intracellular content of MGBG on day 6 of exposure to drug. L1210 cells were incubated in 1.5 µM MGBG or [^{14}C] MGBG for 156 hours before centrifugation, washing and resuspension in fresh medium containing 1.5 µM [^{14}C] MGBG or MGBG, respectively. The intracellular (pellet) content of [^{14}C] MGBG was determined intermittently for 0 - 26 hours after crossover. The "D6 Total" curve (µ) represents the sum of intracellular radioactive MGBG obtained from the influx and efflux experiments.

the influx process can explain the gradual decrease in the intracellular content of MGBG that precedes the recovery of control rates of proliferation.

In Chapter 3, data were presented concerning the relative capacities of putrescine, spermidine and spermine to compete with MGBG for uptake. Since the kinetic characteristics of MGBG uptake had changed upon emergence of resistance, I explored whether or not the K_i's for putrescine, spermidine and spermine had changed also, in order to gain some insight into the nature of the altered transport system. The results are presented in Figure 4-5. The K_i's for each compound had increased in rough proportion to the increase in K_m for MGBG. This finding suggests that the apparent substrate specificity of the transport system in resistant cells had not changed.

When these cells, namely, those exposed to 0.5 μM MGBG for one month, were transferred to drug-free medium, they did not reacquire the capacity of untreated cells to accumulate MGBG even after incubation in drug-free medium for 20 days (Figure 4-6). Kinetic studies were conducted with cells that had been grown in drug-free medium for one month (Table 4-2). By this time V_{max} had returned to control value (321 ± 19 pmole/10^6 cells/hour) but K_m had remained increased (11.57 μM; $P < 0.01$ compared to control cells.

Selection of L1210 Cells Highly Resistant to MGBG

Another approach that I have taken to explore the mechanism of resistance to MGBG involved the selection of L1210 cells presumably resistant on a genetic basis. L1210 cells were incubated in 5 μM MGBG for two weeks and then reseeded in medium containing 10 μM MGBG. A clone of cells retained striking resistance to MGBG even when grown in drug-free medium for months. When grown in drug-free medium, these resistant cells divided slowly, with a doubling time nearly twice that of untreated cells (16 to 20 vs. 8 to 10 hours).

The dose-response curves for the resistant line of cells with MGBG are

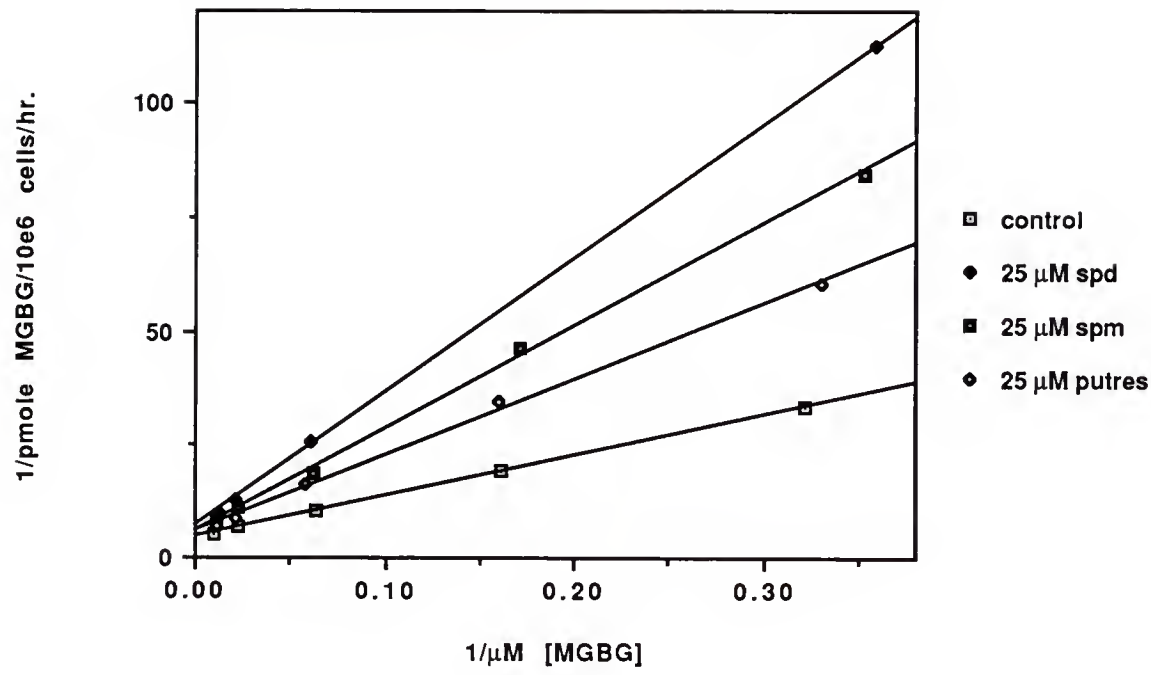

FIGURE 4-5 : Competitive uptake inhibition of MGBG uptake by spermidine, spermine and putrescine in L1210 cells exposed MGBG for 3 weeks. L1210 cells which were exposed to 0.5 μM MGBG for 3 weeks, were kept in drug-free media for 1 week prior to this study. The cells were incubated in the media containing different concentrations of [^{14}C] MGBG (4.0 - 117.5 μM) with or without 25 μM spermidine, spermine or putrescine. The K_i's of spermidine, spermine and putrescine for inhibition of the uptake of MGBG were 7.9 $\pm$ 0.3, 12.0 $\pm$ 0.4 and 20.9 $\pm$ 0.7 μM, respectively.

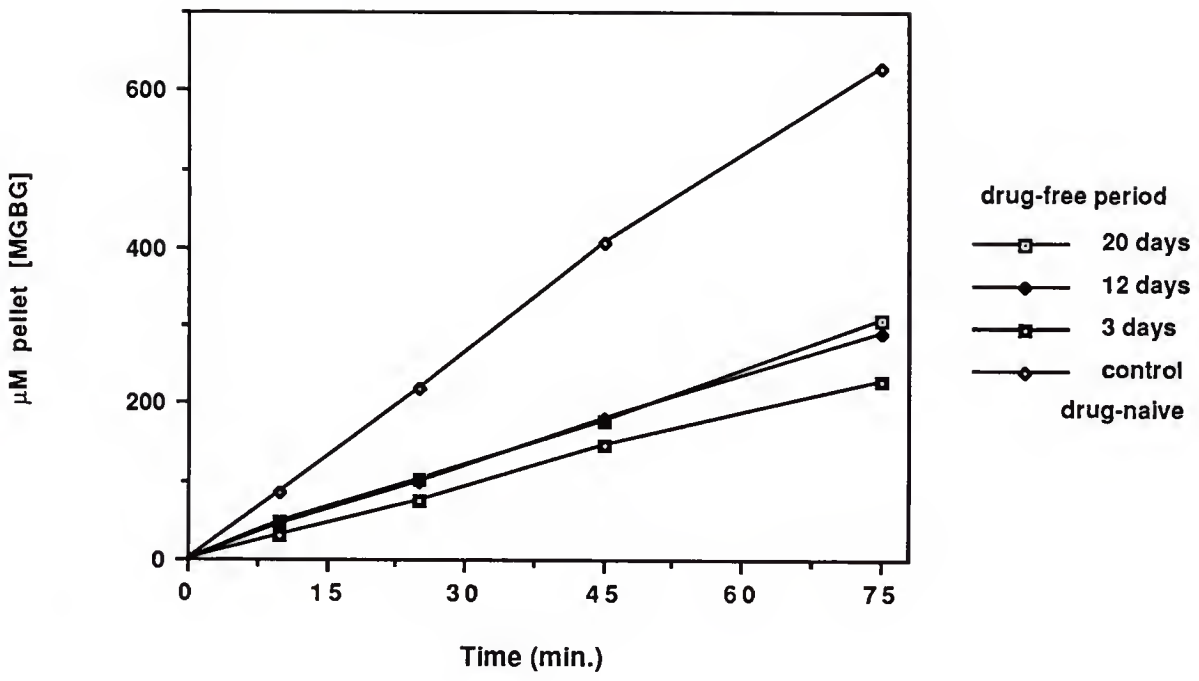

FIGURE 4-6 : Uptake of MGBG in the " recovered " L1210 cells. L1210 cells that had been exposed to 0.5 µM MGBG for one month, were transferred to drug-free medium for 3 - 20 days. These cells were subsequently reseeded in a medium containing [^{14}C] MGBG (20 µM). and the uptake of drug was followed for 75 minutes.

TABLE 4-2 : Kinetics of the uptake of MGBG in untreated L1210 cells, cells that had "recovered" after exposure to MGBG, and a resistant subclone.

Cell Type and Treatment	Km (μM) (mean ± SEM)	Vmax (pmole/10^6 cells/hr.) (mean ± SEM)
Control	4.72 ± 0.17	296 ± 19
Cells exposed to 0.5 μM MGBG for 30 days and then		
- 2 days drug-free	24.87 ± 1.36*	142 ± 13*
- 1 month drug-free	11.57 ± 1.63*	321 ± 19
MGBG-resistant subclone		
- 3 days drug-free	16.18 ± 0.52*	134 ± 15*
- 2 month drug-free	5.14 ± 0.48	145 ± 14*

Note : * $p < 0.01$ (vs control)

presented in Figure 4-7 (growth) and Figure 4-8 (viability). Logarithmically-growing resistant cells were seeded at a starting concentration of 1.05×10^5 cells/ml in media containing 0 to 30 μM MGBG. The cells were reseeded into fresh medium containing MGBG 2 days later. In comparison to the curves depicted in Figures 3-1 and 3-2 with untreated L1210 cells, these cells exhibit striking resistance to MGBG. Even after growth in drug-free medium for six weeks, resistance to MGBG was retained (Figures. 4-9 and 4-10).

Transport Properties of the Resistant Cells

When these resistant cells were exposed to 2.0 to 6.7 μM [^{14}C] MGBG at 37°C, pH 7.40, they accumulated significantly less MGBG than did control L1210 cells (Figure 4-11). This decreased accumulation correlated with an increased K_m (16.2 ± 0.5 compared to 4.7 ± 0.2 μM; $P < 0.01$) and a decreased V_{max} (134 ± 15 compared to 296 ± 19 pmole/10^6 cells/hour; $P < 0.01$). After six-weeks of growth in drug-free medium, V_{max} remained low (145 ± 14 pmole/10^6 cells/hour), the cell doubling time remained long, but K_m had returned to the control value (5.1 ± 0.4 μM).

Once again, the relative affinities toward putrescine, spermidine and spermine of the transport system in resistant cells, as assessed by K_i's for inhibition of MGBG uptake, remained unchanged (Figure 4-12).

Response of multiple drug resistant (MDR) cells to MGBG

Two Chinese hamster ovary (CHO) cell lines were generously provided by Dr. Gurmit Singh (McMaster University, Ontario, Canada). The parental line (Aux B_1) is drug sensitive. The derived line (CH^RC_5) exhibits multiple drug resistant and contains P-glycoprotein. The selection of CH^RC5 is described by Ling and Thompson in1974. Briefly, cross-resistance to a variety of antitumor antibiotics in these CHO cell line was induced by exposure to increasing concentrations of colchicine. This pleotropic or multiple drug, resistance (MDR)

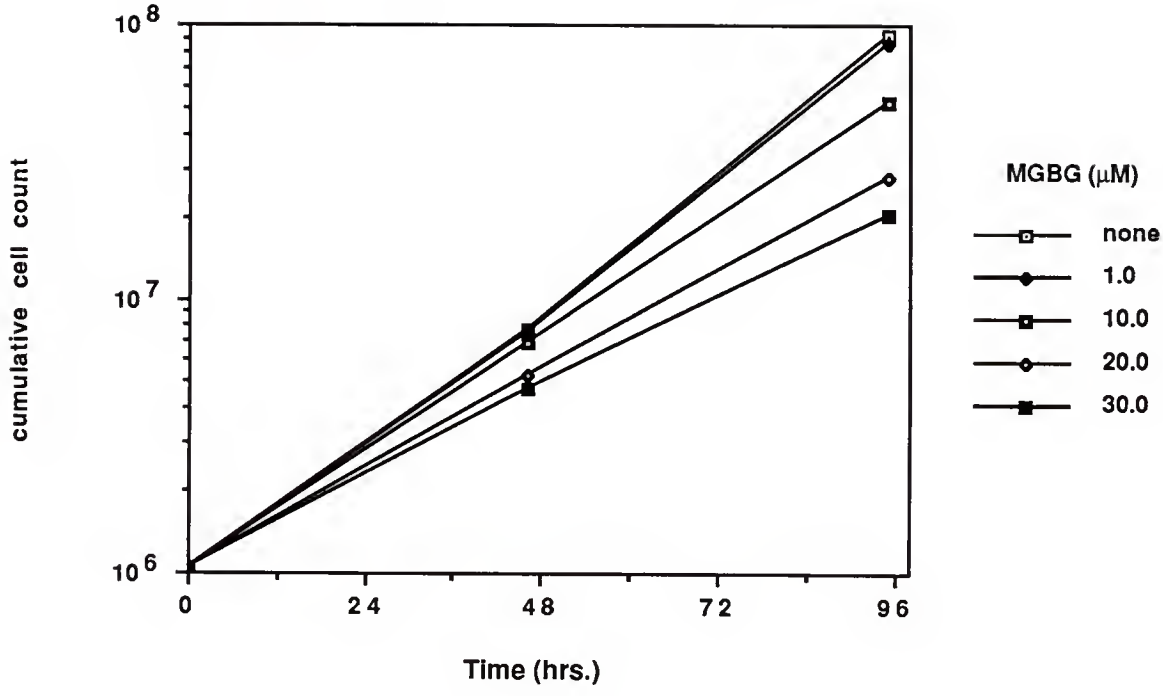

FIGURE 4-7 : Effects of various concentrations of MGBG on growth of MGBG-resistant L1210 cells. Logarithmically-growing resistant cells (see text) were transfered to drug-free medium for 3 days prior to the study. The cells were reseeded at a starting concentration of 1.05 x 10^5 cells/ml in media containing 0 - 30 μM MGBG. The cells were maintained in logarithmic growth by reseeding into fresh medium containing MGBG at 2 days.

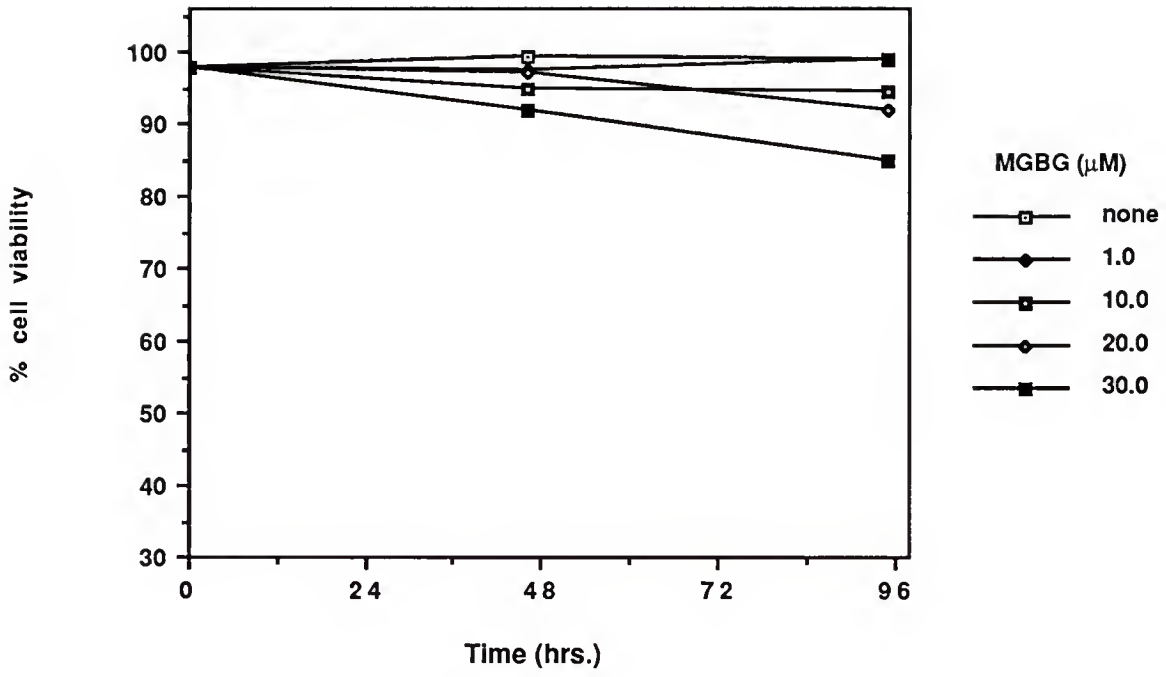

FIGURE 4-8 : Effects of various concentrations of MGBG on viability of MGBG-resistant L1210 cells. See Figure 4-7: viability was assessed by the Trypan Blue dye exclusion method.

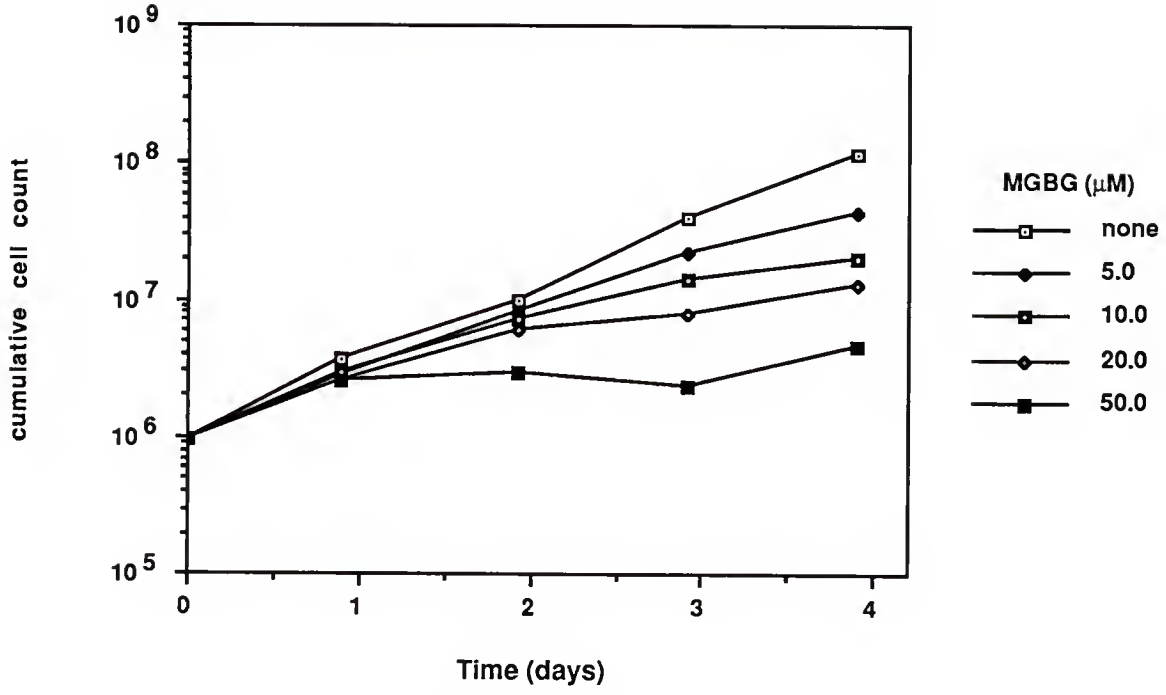

FIGURE 4-9 : Effects of various concentrations of MGBG on growth of the MGBG-resistant L1210 cells after one month of growth in drug-free medium. MGBG-resistant cells (see text) were maintained in drug-free medium for one month prior to the study. Cells in logarithmic growth were seeded in media containing 0 - 50 μM MGBG. Cell counts were determined daily with use of a Coulter Counter and the cells were reseeded into fresh media with or without drug at 2 days.

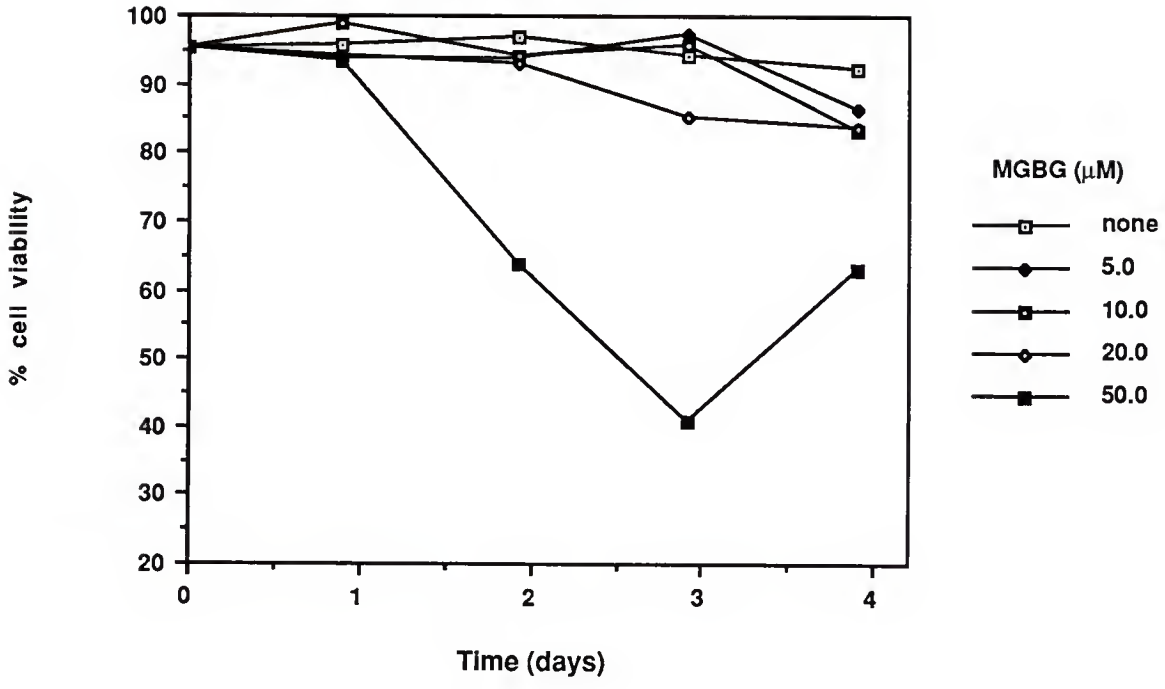

FIGURE 4-10 : Effects of various concentrations of MGBG on viability of the MGBG-resistant L1210 cells after one month of drug-free period. Cell viability was determined daily with use of the Trypan Blue dye exclusion method (see Figure 4-9).

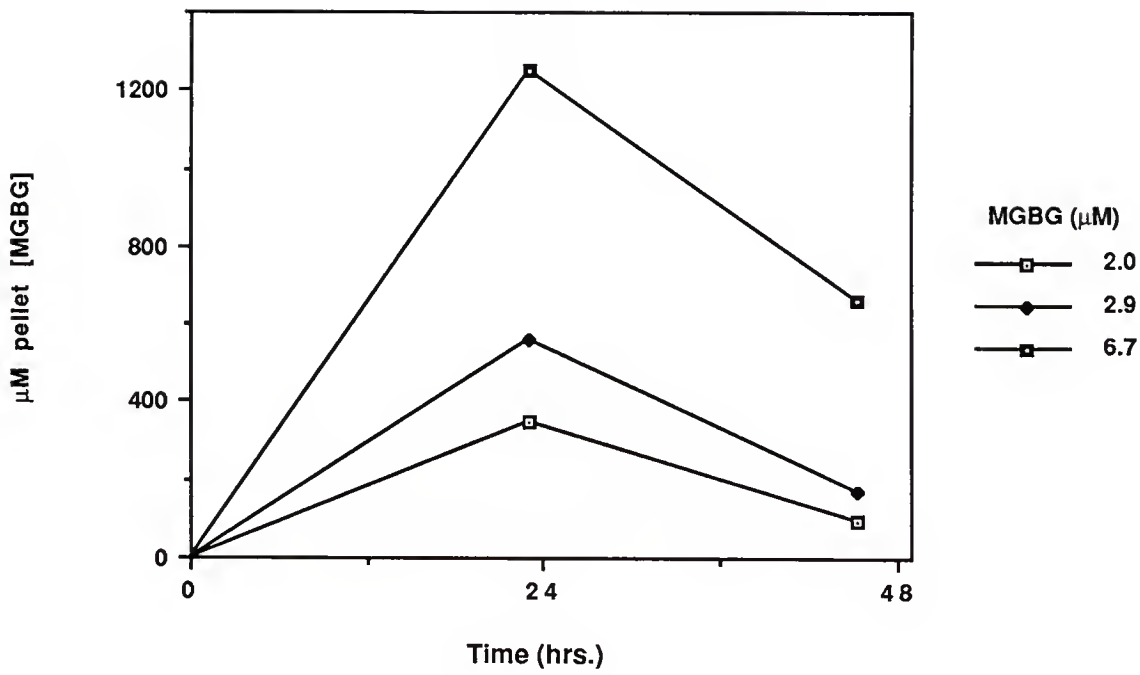

FIGURE 4-11 : Accumulation of MGBG in the MGBG-resistant L1210 cells. The resistant cells (see text) were maintained in a drug-free medium for 3 weeks prior to the study. The logarithmically-growing cells were seeded in various concentrations of [^{14}C] MGBG for 0 - 48 hours and the intracellular content of [^{14}C] MGBG was determined at the times indicated. For comparison, a previously untreated L1210 cell exposed to 1 µM MGBG would be expected to exhibit an intracellular concentration of MGBG of about 2000 µM by 24 hours.

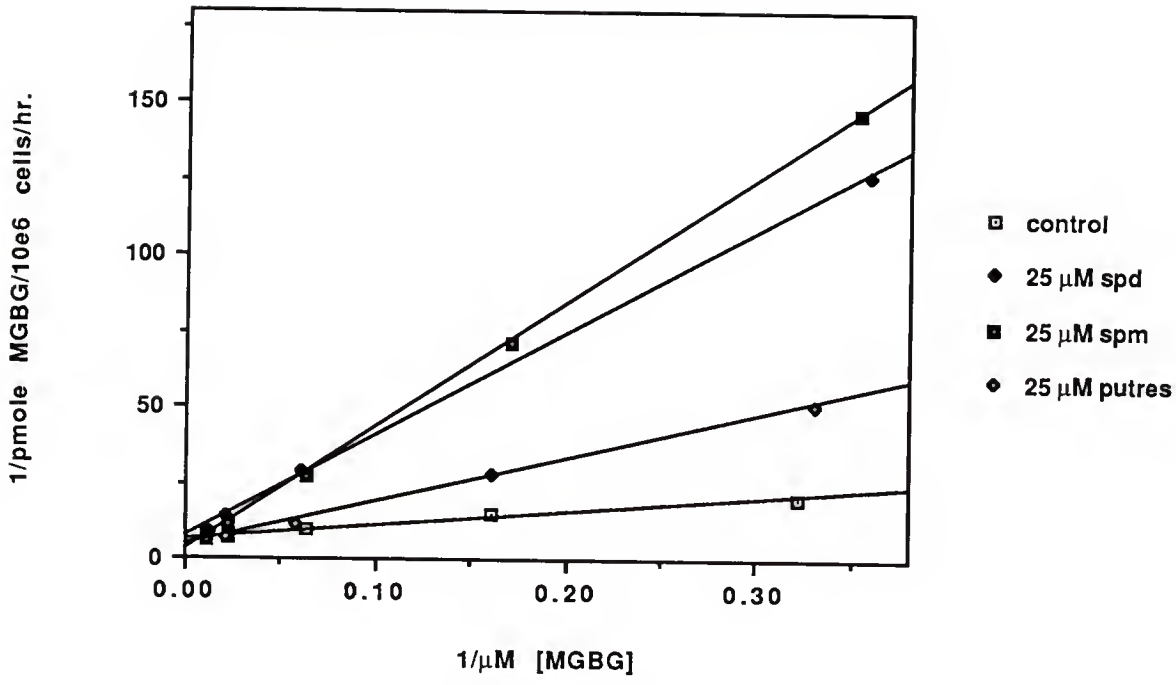

FIGURE 4-12 : Competitive inhibition of MGBG uptake by spermidine, spermine and putrescine in MGBG-resistant L1210 cells. MGBG-resistant L1210 cells were kept in drug-free medium for 1 week prior to this study. The cells were incubated in the media containing different concentrations of [^{14}C] MGBG (4.0 - 117.5 μM) with or without 25 μM spermidine, spermine or putrescine. The K_i's of spermidine, spermine and putrescine for inhibition of the uptake of MGBG were 2.68 ± 0.09, 2.51 ± 0.08 and 9.33 ± 0.10 μM, respectively.

was associated with the presence of a 170 KDa membrane glycoprotein (Juliano and Ling, 1976). This protein is termed the P-glycoprotein because of its association with the plasma membrane. It behaves as an efflux pump and hence, decreases intracellular accumulation of antineoplastic agents in resistant cells. Both CHO cell lines were grown in alpha-modified essential medium (alpha-MEM) with L-glutamine, ribonucleosides and deoxyribonucleosides (GIBCO Laboratory). The medium was supplemented with 10% fetal bovine serum (FBS), streptomycin and penicillin. The cells were grown in Petri dishes and incubated at 37°C in 5% CO_2 : 95% air. The sensitive and MDR CHO cell lines were exposed to different concentrations of MGBG (0 - 10 μM) for 24 to 72 hours. The initial cell number plated was such that the control cells would not reach confluence before day 3, when the inhibitory effect of the drug was evident. After 46 or 72 hours of MGBG exposure, the medium was removed and the cells were trypsinized. Trypsinization was stopped by adding two volumes of medium containing 10% FBS. The dishes were gently shaken to dislodge the cells and repipetted to break up any clumps before counting the cells by a Coulter Counter. Cell viability was determined with use of trypan blue dye exclusion method.

Due to the fact that the doubling time of the Aux B_1 cells is shorter than the CH^RC_5 cells, the sensitivities to MGBG of these two cell lines were compared after exposure for approximately 3 doubling times (46 and 72 hours for Aux B1 and CH^RC_5 cells, respectively). The results depicted in Figure 4-13 reveal that there is no difference in the sensitivity of the CH^RC_5 and the Aux B1 to the antiproliferative activity of MGBG. This finding suggests that different mechanisms of resistance exist in the MDR and the MGBG-resistant cells.

Correlation between Transport and Resistance

It is important to ask whether or not "all" of the resistance to MGBG that I

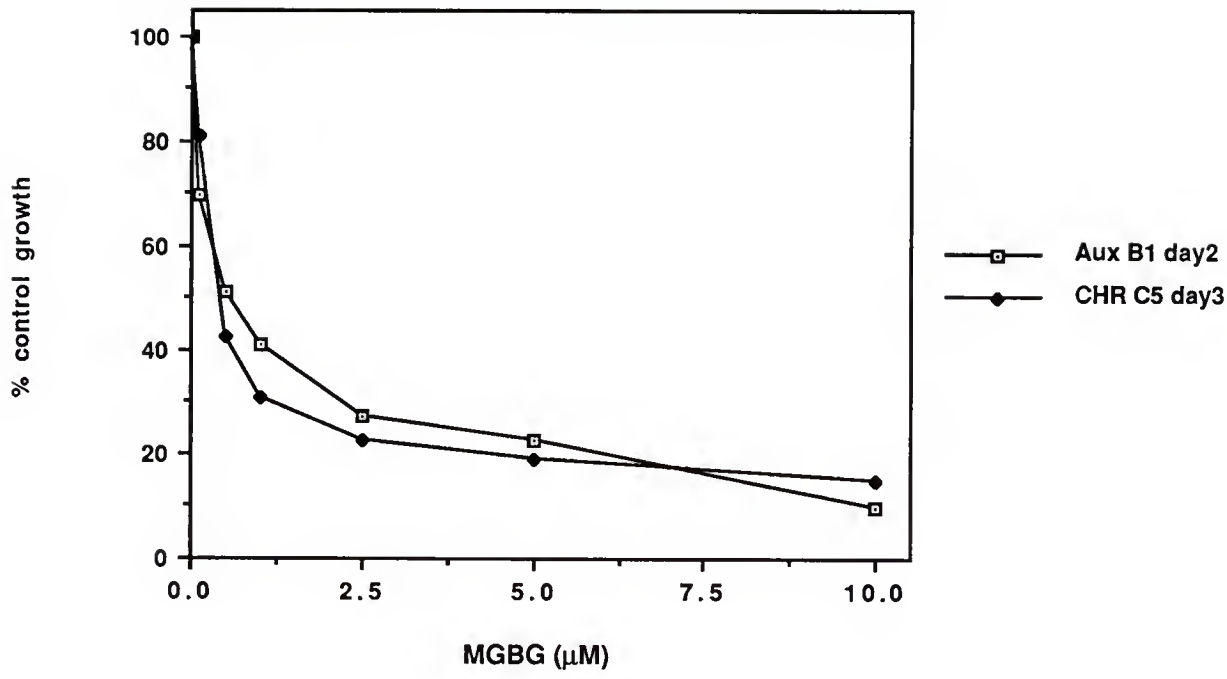

FIGURE 4-13 : Comparison of MGBG dose-response curve of parental drug-sensitive (Aux B1) and multidrug resistant (CH^RC_5) cell lines. The two CHO cell lines were exposed to different concentrations of MGBG (0 - 10 μM) for 0 - 3 days (2 days for Aux B_1 cell line and 3 days for CH^RC_5 cell line). Cell counts were determined by electronic particle analysis (Coulter Counter) and cell viability was determined with use of the Trypan Blue exclusion method. The results are expressed as percent control growth (no drug).

observed in various experiments, can be attributed to changes in transport of MGBG (i.e., in the accumulation of drug within cells). I have attempted to approach this question by comparing untreated cells, "adapted" cells (0.5 μM MGBG for one month) and selected cells (as defined above) with regard to intracellular concentration of MGBG and rates of growth. There was a linear relationship ($r = 0.91$) between intracellular concentration of MGBG at 24 hours and growth rate over the following day, regardless of the type of cell involved (Figure 4-14). in other words, the data from all cells fit the same line. Growth rates between 0 and 48 hours have also been analyzed with similar results. These results indicate that virtually all of the resistance that develops can be explained by changes in transport and not by some intrinsic change in sensitivity to MGBG within cell.

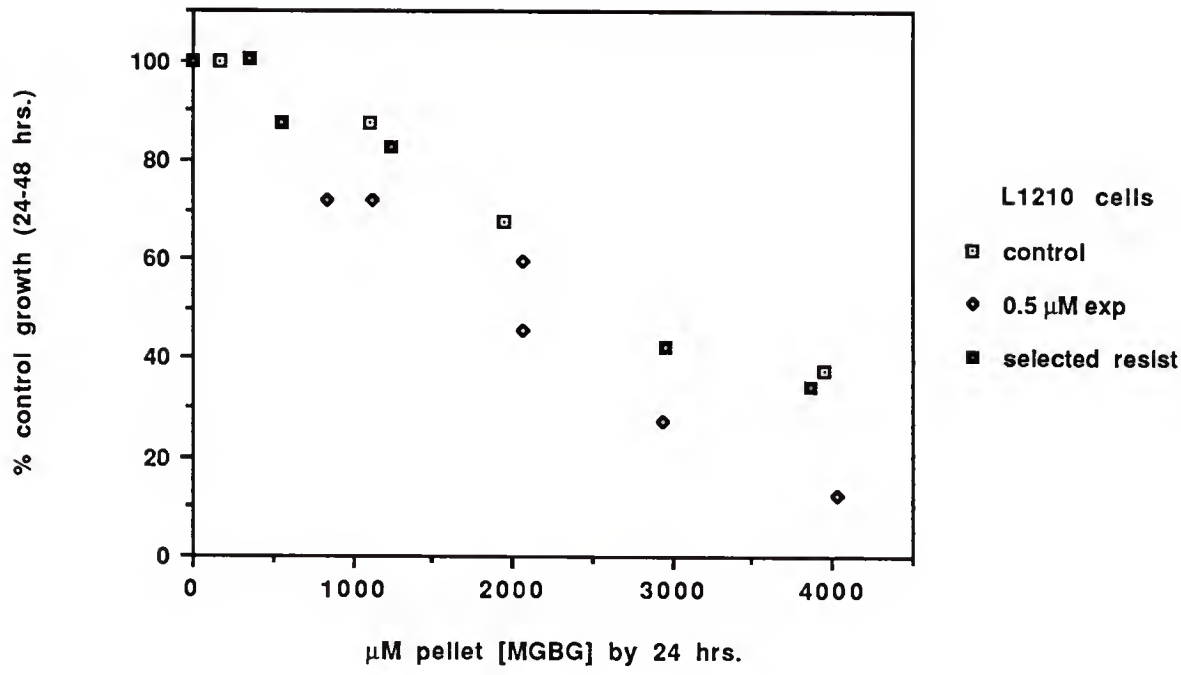

FIGURE 4-14 : Correlation between intracellular content of MGBG and the rate of cell proliferation in various type of L1210 cells. Untreated L1210 cells, L1210 cells that "recovered" after exposure to 0.5 µM MGBG, and MGBG-resistant subclone (see text), were exposed to various concentrations of [^{14}C] MGBG for 0 - 48 hours. The intracellular content of [^{14}C] MGBG was determined at 24 hours. Cell counts were determined at 0, 1 and 2 days with use of a hemacytometer, and cell viability was determined by Trypan Blue dye exclusion method.

CHAPTER 5
DISCUSSION

Discussion will focus on: 1) the mechanism of MGBG transport, and 2) resistance of L1210 cells to MGBG.

Mechanism of MGBG Transport

As reported earlier (Field et al., 1964, Dave and Caballes, 1973, and Porter et al., 1981), MGBG was found to accumulate extensively in the cell pellet. The extensive accumulation of MGBG we observed, was not due to surface binding since the radiolabelled MGBG in the cell pellet could not be displaced by 1 mM MGBG. The fact that nongrowing L1210 cells do not accumulate MGBG also makes significant adsorption of MGBG to the cell surface most improbable as an important quantitative factor in accumulation. All of our experiments suggested that the major gradient in MGBG concentration was across the plasma membrane. Accumulation of MGBG inside the cells was time-dependent, and the rate of accumulation was linear with time for more than 20 hours. Interestingly, accumulation of the drug over 20 hours could be predicted by kinetic constants from 20-minute experiment (Table 3-1).

Reported effects of MGBG on mitochondrial structure and bioenergetic functions were described in Chapter 1. Even though mitochondria seem to be an important site of MGBG's action, studies with use of isolated mitochondria and digitonin revealed that mitochondria were not the driving force for extensive

accumulation of MGBG inside the cells. In other words, there was no suggestion of an appreciable gradient of MGBG between cytosol and mitochondrial compartments. Most of the intracellular drug was likely to be in the cytosol. The actual free or unbound concentration of MGBG in the cytosol or mitochondria remains unknown. MGBG is a weak base with pK_a's of about 7.5 and 9.2 at 25 °C (Wiliams-Ashman and Seidenfeld, 1986). At physiological pH, MGBG exists primarily as a mixture of monovalent and divalent cations, and a large number and wide variety of binding sites likely exist intracellularly. As noted above, the accumulation of MGBG was linear for at least 20 hours; this result suggests the binding of MGBG to intracellular binding sites. Our experiments with digitonin to permeabilize the cell membrane, however, revealed an immediate release of the labelled drug with cytosolic components, a result which suggests that MGBG was loosely bound to the intracellular binding sites at least those associated with membranes or organells. Efflux of MGBG was close to first order (Figure 3-8), another finding which implied that most of the intracellular MGBG was either loosely bound or dissociated from binding sites at an appreciable rate. In addition, because MGBG is relatively poorly protonated at physiological pH compared to the natural polyamines, MGBG would not likely bind as tightly as spermidine or spermine to anionic polynucleotides and phospholipids or other intracellular molecules. The extremely low abundance of S-adenosylmethionine decarboxylase (AdoMetDC), diamine oxidase and spermidine/spermine N'-acetyltransferase which are high affinity binding sites for MGBG, would preclude these enzyme from binding more than a negligible fraction of the MGBG that is found within the cells (Williams-Ashman and Seidenfeld et al., 1986).

As mentioned earlier, MGBG is positively charged under physiological conditions. It is unlikely that MGBG enters the cells by simple diffusion. Indeed,

the influx of MGBG exhibits saturation kinetics with apparent K_m's of 2.10 ± 0.08 and 9.34 ± 1.04 μM, for the sodium-dependent and total processes, respectively. Thus, it seems that the uptake of MGBG involves a carrier with reasonably high affinity for MGBG. This carrier is likely also to be involved in the transport of the naturally occurring polyamines, spermidine and spermine, as well as putrescine, in that each of these compounds competed with MGBG for uptake. This competition had been recognized previously (Dave and Caballes, 1973, Janne et al., 1978, Porter et al., 1981, Williams-Ashman and Pegg, 1981, and Heby, 1981). Studies of countertransport also support the notion of a carrier molecule. Interpretation of this experiment, however, is complex because of the existence of naturally occurring polyamines within the cells. The rates of uptake of MGBG were strongly influenced by pH (Figure 3-6). An increase in extracellular hydrogen ion concentration enhanced the rate of uptake of MGBG. These results suggested that the divalent form of MGBG ($MGBG^{2+}$) was the actual substrate for transport into the cells. This hypothesis was supported further by the good agreement between the observed rate of uptake and a rate predicted using values of $MGBG^{2+}$ concentration based on Henderson-Hasselbach computations (Table 3-2).

Even though normal and malignant cells in active growth could accumulate MGBG (Field et al., 1964, Kramer et al., 1985), an intracellular concentration 2000 fold higher than that in the medium concentration was observed in our studies (Figures 3-4 and 4-1). This result does not distinguish active transport from facilitated diffusion coupled with intracellular binding. To pursue that issue, studies were conducted to determine whether energy was required for the accumulation of MGBG. The uptake of MGBG at low temperature (0 - 4°C) was only about 10% of that of uptake at 37°C. Preincubation of cells with inhibitors of energy metabolism (antimycin A, KCN and 2,4-DNP) decreased

and 2,4-DNP) decreased intracellular accumulation of MGBG. These results would be anticipated if energy was required for the accumulation of the drug. In addition, efflux of MGBG was also affected by temperature. At 0 - 4 °C, the efflux of MGBG was barely detectable. It is often difficult to define the energy requirement for active transport because of the potential nonspecific effects of inhibitors of ATP synthesis.

The transport of sugars, amino acids, and numerous other solutes in many cell types is observed to be driven against a chemical potential gradient by cotransport with sodium ion down its electrochemical potential gradient. The free energy dissipated by sodium ion movement down this gradient is coupled with the increase in free energy associated with concentration of the substrate within the cell. In various systems of this type, the carrier is proposed to be a two-site molecule; one site accommodating sodium ion and the other simultaneously accomodating the transported substrate (Schafer and Barfuss, 1986). We found that transport of MGBG in L1210 cells was sodium-dependent. The sodium-dependent uptake process was consistent with a single transport system in that the Lineweaver-Burk plot for sodium-dependent uptake was linear. The dependency of MGBG uptake on sodium was rather specific because the uptake of MGBG decreased when sodium was replaced by choline, lithium, or especially, potassium or cesium. Even though there was a decrease in the uptake of MGBG when sodium was replaced by choline, there was a substantial amount of the intracellular accumulation of the drug. A comparison of kinetics in these circumstances revealed that the presence of sodium ion did not affect V_{max} but did decrease K_m or increase affinity toward MGBG. The effect of sodium was therefore particularly important at low (μM) concentrations of MGBG.

If the sodium electrochemical gradient is involved in MGBG transport,

dissipation of this gradient with specific antibiotic ionophores should inhibit uptake of MGBG. The results summarized in Table 3-5 suggested that this was indeed the case. The ion selectivity of ionophores is a combined function of the energy required for desolution of the ion and the liganding energy obtained on complexation (Simon and Morf, 1973 and Eisenman et al., 1968). Valinomycin which exhibits 10,000 : 1 preference for K^+ (radius, r = 1.33 °A) over Na^+ (r = 0.95 °A) in both biological and model system (Moore and Pressman, 1964, Pressman, 1965, 1968, 1976), has no effect on the intracellular accumulation of MGBG in L1210 cells. Monensin, which is more specific for Na^+ than K^+, exhibited a moderate inhibition of MGBG accumulation whereas Gramicidin A, a channel-forming ionophore which does not discriminate between Na^+ and K^+, was the most potent inhibitor of MGBG uptake. The inhibitory effect of monensin and gramicidin A implied the requirement of an electrochemical potential gradient and the possible co-transport of Na^+ with MGBG to enter the cells. Although there was no direct evidence to support the cotransport of sodium ion with MGBG in our studies, the fact that MGBG accumulated more than 2000-fold within the cell, and the inhibitory effects of sodium ionophores, were strongly suggestive. A23187 also caused a decrease in intracellular MGBG accumulation. This compound is a carboxylic ionophore which possesses a high selectivity for divalent over monovalent ions (Pfeiffer et al., 1974), allows Ca^{++} to enter cells (Reed and Lardy, 1972, Pressman, 1973, Pressman and deGuzman, 1974,1975) and is capable of stimulating various Ca^{++}-dependent reactions without disturbing preexisting balances of Na^+ and K^+. The inhibitory effect of A23187 on MGBG uptake might be due to the biological effects of high intracellular level of Ca^{++}. A23187 also transports Mg^{++}, but gradients of this ion across biological membranes seldom participate in biological control (Pressman, 1976). Interpretation of the specific effects of ionophores is very

complex.

Resistance of L1210 Cells to MGBG

Studies in drug resistant tumor cell lines have identified many different mechanisms for the development of resistance. In cell culture studies, three major mechanisms of genetic resistance have been delineated and characterized. These mechanisms include overproduction of the drug target (e.g., gene amplification, Schimke et al., 1983, Rice et al., 1987, Somfai-Relle et al., 1984, and Jones, 1984), reduced drug permeability (Mandel and Flintoff, 1978, Sirca et al., 1987, Rodrigues et al.,1987, Waud, 1987, and Kraker and Moore, 1988) and altered target interaction of the drug (Herman et al., 1979, Schabel et al., 1982, Seeber et al., 1982, Hunt et al., 1983, and Horns et al., 1983). Murine leukemia L1210 cells in our studies acquired a drug resistant phenotype to MGBG during exposure to sublethal concentrations of drug. Our data suggest that a decrease in drug uptake was responsible for this resistance. We found that the influx rate of MGBG in L1210 cells started to decrease significantly within a few days of exposure whereas the rate of efflux remained unchanged. A separate subclone of genetically stable MGBG-resistant L1210 cells also exhibited a deficiency in drug transport which was characterized by a significant decrease in V_{max} and an increase in K_m for MGBG transport. In both "adapted" cells and the MGBG-resistant subclone, as well as in previously untreated cells, an apparent intracellular concentration of MGBG less than 400 μM was required for toxicity. A similar quantitative relationship between the intracellular concentration of MGBG 24 hours after exposure and the growth rate over the following day was observed for all types of L1210 cells. It is therefore unlikely that an intracellular response to a given concentration of MGBG plays a significant role in the development of resistance. MGBG had been reported to be metabolically inert (Warrel and Burchenal, 1983), and we

have confirmed that finding.

Two sites of MGBG action have received most attention with regard to its antiproliferative activity: mitochondria and polyamine metabolism (inhibition of AdoMetDC). Of these, as mentioned earlier, it appears that drug effects related to mitochondrial integrity correlate best with the antiproliferative action of MGBG in various cell lines (Pleshkewych et al., 1980 , Mikles-Robertson et al., 1979 and 1981, Corti et al., 1974, Pathak et al., 1977, Wiseman et al., 1980 and 1983, and Porter et al., 1979). In general, drug effects on parameters related to polyamine metabolism fail to correlate with the antiproliferative action of MGBG. In addition, there is no simple correlation between direct inhibition of AdoMetDC and antileukemic activity in vivo for a series of MGBG analogs (Mihich, 1975, Corti et al., 1974, and Porter et al., 1981). Prolonged exposure to MGBG actually increased activity of AdoMetDC. This might be related to an increase in the enzyme synthesis (Pegg et al., 1973, Pegg, 1984, and Fillingame and Morris, 1973) or its stabilization (Williams-Ashman and Seidenfield, 1986).

I explored the relationship between multiple drug (MDR) resistance and resistance to MGBG. Studies of Chinese hamster ovary (CHO) cells that were resistant to the drug, colchicine (Ling and Thompson, 1974 and Ling, 1975), revealed a specific alteration of the plasma membrane in multidrug resistance. Drug-resistant cells contained a unique glycoprotein that was absent in the drug-sensitive cells (Juliano and Ling, 1976). This glycoprotein was large in size (molecular weight, about 170,000) and was named P-glycoprotein for its association with the apparent permeability barrier at the plasma membrane to drugs that were included in multidrug resistance. Further evidence suggested that P-glycoprotein functions as a drug efflux pump (Gros et al., 1986 and Chen et al., 1986). Interestingly, resistance of MDR cells to colchicine, adriamycin and other agents can be reversed by exposure to calcium channel-blocking

agents, a procedure which also reverses the defect in intracellular drug accumulation (Tsuruo et al., 1982, Rogan et al., 1984, and Willingham et al., 1986). Due to the fact that the efflux rate of MGBG in L1210 cells in our studies remained unchanged after adaptation to MGBG, it is unlikely that resistance to MGBG would be an MDR-type resistance. When parental and MDR CHO cell lines were exposed to different concentrations of MGBG, both cell lines were equally sensitive to the antiproliferative activity of the drug.

Our results were discussed earlier in terms of "adaptation" for the L1210 cells which had recovered within two weeks after exposure to 0.3 - 5 μM MGBG. Several lines of evidence from our studies suggest that this resistance to MGBG was due to adaptation of the cells to the drug, not selection of preexisting mutant. In recent years, it has become increasingly evident that neoplasms contain complex subpopulations of cells, each of which differs in expression of biological properties. A concept now receiving increased attention is that drug resistant neoplastic stem cells often arise from mutations, and that the drug-resistant phenotype is inherited and propagated. The concept of the development by spontaneous mutation of cancer cells that have resistance to drugs they not yet encountered is called Goldie-Coldman hypothesis (Goldie and Coldman, 1979). According to the Goldie-Coldman model, rates of mutation give rise to resistance to a variety of antineoplastic agents in a variety of mammalian tumor cells at a prevalence of 1 per 10^4 to 10^7 cells. The fact that MGBG uptake by cells "adapted" to the presence of 0.5 μM had not returned to the control transport rate even after removal of cells from drug for six weeks raised the possibility that we had selected a mutant cells rather than caused an "adaptation" of most cells. If we assume that only mutant cells can divide during the course of MGBG exposure, the Y-intercept obtained from an extrapolation of the MGBG dose-response curve of L1210 cells would reveal the frequency of

preexisting MGBG-resistant cells in overall cell population. At 0.5 μM MGBG, this frequency was observed to be 1 in 10^4 cells (Figure 3-1), a value within the estimated range denoted above. However, clonogenic experiments (Figure 3-3) revealed that a large fraction of cells (> 60 %) was clonogenic even 72 hours after exposure to 0.5 μM MGBG. In addition, the intracellular content of MGBG had decreased significantly within a few days of initial exposure to the drug. These results suggest strongly that most cells were adapting to the presence of MGBG as opposed to, or at least in addition to, the selection of preexisting mutants of L1210 cells that were constitutively deficient in MGBG transport. In other words, the concept of preexisting mutant cells is unable by itself to explain the sudden and significant decrease in the intracellular content of MGBG within a few days after exposure to the drug. The importance of adaptive regulation of transport is also supported by the kinetic results obtained midway through the change in transport (after 6 days of incubation with drug). Had two types of cells been present at this time (one with a K_m of 4.72 μM and the other with a K_m of 24.87 μM for MGBG influx), I would have anticipated a nonlinear Lineweaver-Burk plot; no hint of nonlinearlity was observed. Furthermore, a comparison of the cells "adapted" to MGBG and those selected for resistance to MGBG suggests that both types of cells exhibit an adaptive increase in a Km, but a decrease in V_{max} is characteristic only of the genetically stable subclone and not of the "adapted" cells.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Summary

We have studied the effect of MGBG on cell proliferation in murine leukemia L1210 cells. Because these L1210 cells developed resistance to MGBG during exposure, our experiments were designed to define mechanism of resistance to MGBG in these cells and also in a clone of L1210 cells selected for resistance to a lethal concentration of MGBG.

The important role of transport in the development of resistance to MGBG, was first implied by a study of the apparent intracellular concentration of MGBG for 12 days after exposure of previously untreated L1210 cells to drug. We confirmed that MGBG accumulated quite extensively in these cells. The apparent intracellular concentration of MGBG depended on both the concentration of drug added to the medium and the duration of exposure. At 1 μM MGBG in the medium, the intracelllular concentration of drug reached approximately 2000 μM about 24 hours after the addition of MGBG. Despite continued presence of the drug in the medium, the intracellular concentration of MGBG decreased to approximately 500 μM by day 6 and further to 100 - 400 μM thereafter. The same pattern applied to all doses of MGBG as long as they were high enough to affect cell growth; peak intracellular concentrations occurred 24 - 48 hours after the start of incubation and invariably decreased by 72 hours. Decrease in the intracellular accumulation of MGBG was due to a

decrease in the influx, not an increase in the efflux of MGBG. The rate of cell proliferation returned to the control value within two weeks of exposure, a time when the intracellular concentration of MGBG was < 400 μM. The dose-response (cell proliferation) curve of these L1210 cells was shifted to the right more than 10 fold. The lethal concentration of MGBG increased from 5 to about 50 μM in these "adapted" L1210 cells. Similar results were obtained in studies with a subclone of genetically stable MGBG-resistant L1210 cells. Higher extracellular concentrations of MGBG were required for the L1210 resistant subclone to achieve a given intracellular concentration of MGBG. "Adapted" L1210 cells (0.5 μM MGBG for 1 month) and MGBG-resistant subclone, as well as untreated L1210 cells, were compared with regard to intracellular concentration of MGBG and rate of cell growth. There was a linear relationship between intracellular concentration of MGBG at 24 hours and rate of cell proliferation over the following day, regardless of the type of cell involved. At apparent intracellular concentration in excess of 400 μM, the growth rate of all cells decreased.

Indirect methods were used to determine the intracellular distribution of MGBG in L1210 cells. Mitochondria were unlikely to be the driving force for an extensive intracellular accumulation of MGBG even though the effect of MGBG was specific for mitochondria.

Transport mechanism of MGBG was also examined. The transport of MGBG seemed to be energy dependent and carrier-mediated. It exhibited saturable kinetics with a V_{max} and K_m of 296 ± 19 pmole/10^6 cells/hour and 4.72 ± 0.169 μM, respectively. The rate of transport depended on temperature, pH and rate of cell proliferation. Inhibitors of energy metabolism, ionophores, and the replacement of sodium ion by other cations, all significantly decreased the rate of accumulation of MGBG. The experiments with ionophores suggested that

uptake of MGBG depended on a plasma membrane electrochemical gradient. The facilitation of uptake by sodium ions was compatible with a process in which sodium increased the affinity of the carrier for MGBG, but two separate processes, sodium-dependent and sodium-independent remain possible.

Resistance to MGBG was not associated with MDR-type resistance because the sensitivity of the parental and MDR (containing P-glycoprotein) CHO cell lines to MGBG, was indistinguishable.

Future Directions

Since transport of MGBG is the major determinant of sensitivity, and since the MGBG gradient occurs at the plasma membrane, future directions focus use of membrane vesicle technique to further characterize the transport of MGBG. There are several interesting analogies with other known transport system, e.g. for amino acid (Rinehart and Chen, 1984 and Karl et al., 1989) and/or some organic cations which was resently reported by Sobol (1989), that should be explored. It would also be of interest to examine the sodium dependency of MGBG transport in the two types of resistant cells, "adapted" and MGBG-resistant subclones. Understanding of the mechanism of regulation of the MGBG transporter may lead to a discovery of new strategies for cancer chemotherapy, either with MGBG or with novel analogs.

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